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Studies on the Destruction of Red Blood Cells. VI. The Spleen as a Source of a Substance Causing Agglutination of the Red Blood Cells of Certain Patients With Acquired Hemolytic Jaundice by an Antihuman Serum Rabbit Serum (Coombs' Serum). Philip F. Wagley, M.D., Shu Chu Shen, M.D., Frank H. Gardner, M.D., and William B. Castle, M.D., Boston, Mass. 1197

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STUDIES ON THE DESTRUCTION OF RED BLOOD CELLS

VI. The Spleen as a Source of a Substance Causing Agglutination of the Red Blood Cells of Certain Patients With Acquired Hemolytic Jaundice by an Antihuman Serum Rabbit Serum (Coombs' Serum)

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INTRODUCTION

Boorman, Dodd, and Loutit[1] have demonstrated that the immune serum prepared by Coombs, Mourant, and Race[2] by the injection of human serum (not red cells) into rabbits may be used to distinguish certain types of acquired hemolytic jaundice from congenital hemolytic jaundice. They observed that the washed red blood cells from five patients with acquired hemolytic jaundice were agglutinated by this serum. On the other hand, the washed red blood cells from seventeen patients with congenital hemolytic jaundice were not agglutinated. The test was originally developed by Coombs and associates[3] for the detection of the incomplete Rh agglutinin on the supposition that such sensitized red blood cells might carry adsorbed antibody globulins. The red blood cells are agglutinated presumably as a physical manifestation of the union of the globulin or other substance on the surface of the red cells with the antibody in the test serum.

Acquired hemolytic jaundice has been reported as an independent acute or chronic entity[4, 5] or in association with malaria, tuberculosis, cirrhosis of the liver, Hodgkin's disease, leucemia, carcinomatosis, and cysts and tumors of the ovary.[6, 7] Splenomegaly is often present in such conditions, and splenectomy in some instances, although not necessarily altering the cause of the disease, may be beneficial by decreasing red blood cell destruction. In some cases the agglutination titer of the patient's red blood cells in antihuman serum rabbit serum declines after splenectomy.[8] Such observations, also encountered in our own experience, have raised the question of whether the spleen, which contains both lymphocytes and reticulo-endothelial cells as potential sources of antibodies, might be a source of the substance presumably adhering to the red blood cells of these patients. The principal objective of the experiments reported here was to examine the spleens removed from patients with acquired hemolytic jaundice and various other conditions for the presence of substances possessing an affinity for normal red blood cells.

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METHODS

All spleens were obtained at operation and unless immediately employed in the experimental procedure were frozen at −4° C. and preserved at that temperature. Spleens from three patients with acquired hemolytic jaundice and from two patients with thrombocytopenic purpura were studied immediately after splenectomy. Spleens from one patient with acquired hemolytic jaundice, three with congenital hemolytic jaundice, two with thrombocytopenic purpura, three with congestive splenomegaly (Banti's syndrome), and two with Gaucher's disease were studied after being frozen for from one to two years.

A portion of each spleen was finely ground with a domestic meat chopper. The ground pulp obtained from the spleens that were studied immediately after splenectomy was then stirred vigorously with approximately twice its volume of distilled water. The distilled water was then allowed to drain briefly from the pulp through gauze. This procedure was repeated three times, at which point the pulp usually contained no microscopically visible intact red blood cells. Then the splenic pulp was resuspended in normal saline solution which was finally removed by draining through gauze. The pulp was then weighed. In the case of the frozen spleens the effect of the previous freezing and thawing served to destroy the red blood cells present and so rendered treatment with distilled water unnecessary. The absence of red blood cells from the pulp of these spleens was confirmed in each instance by inspection of a small sample under the microscope.

A sample of pulp from each spleen was then incubated for from one to two hours at 37° C. with 3 c.c. of a 40 per cent suspension of either normal Group 0 Rh-negative red blood cells or of red blood cells of the patient's blood group. The red blood cells had previously been carefully washed three times with physiologic salt solution. The amount of splenic pulp employed in each incubation was judged by the amount that was shown not to cause significant hemolysis of the washed red blood cells during a preliminary period of incubation of one hour at 37° C. With the fresh spleens, 12 Gm. of pulp were employed. With the previously frozen and thawed spleens, from 1.5 to 12 Gm. of pulp were used. After the incubation period, two or three times the volume of physiologic salt solution was added to the mixture which was then stirred and filtered through gauze one or more times in order to separate the red blood cells from the splenic pulp. The red blood cells obtained were then carefully washed three times with normal physiologic salt solution and finally made up into a 5 per cent suspension in that medium. A control sample of red blood cells was treated in a similar fashion except that it was not added to splenic pulp.

The test for the presence of adsorbed substance was carried out by incubating for one hour at 37° C. 0.2 c.c. of the 5 per cent suspension of red blood cells with 0.2 c.c. amounts of serial dilutions in physiologic salt solution of an antihuman serum rabbit serum. The rabbit serum was prepared according to the method of Coombs, Mourant, and Race.[2] Its strength was determined by a modification of the method of Haberman and Hill[9] against a suspension in physiologic salt solution of Rh-positive red blood cells which had previously been sensitized with an Rh blocking antibody with a titer of 1:64. The rabbit serum caused microscopic agglutination of the washed cells sensitized with the blocking antibody in a dilution of 1:128.*

At the conclusion of the incubation period each red blood cell suspension was studied microscopically for evidence of agglutination. The control sample of red blood cells was handled in the same fashion except that its agglutinability was determined only with the undiluted test serum. In addition, tests were made of the agglutinating power of the antihuman serum rabbit serum against washed red blood cells derived from the peripheral blood and from the splenic pulp of the four patients with acquired hemolytic jaundice at the time of splenectomy.

RESULTS

Inspection of the data shown in Table I indicates that subsequent to incubation with the splenic pulp from the four patients with acquired hemolytic jaundice, normal washed red blood cells, either Group 0 Rh negative or belonging

*It is essential that the serum employed in testing the effects of incubation with splenic pulp has a relatively high titer.

to the same blood group as that of the patient, were agglutinated by the antihuman serum rabbit serum. Though definite when observed under the microscope, in no instance was the agglutination present when the serum used was diluted. On the other hand, normal red blood cells after similar incubation with the splenic pulp from patients with congenital hemolytic jaundice were

TABLE I. THE AGGLUTINATING EFFECTS OF ANTIHUMAN SERUM RABBIT SERUM (COOMBS' SERUM) UPON WASHED NORMAL RED BLOOD CELLS SUBSEQUENT TO INCUBATION WITH THE PULP OF SPLEENS DERIVED AT OPERATION FROM PATIENTS WITH VARIOUS DISEASES

CASE	DISEASE	METHOD OF PREPARATION OF SPLENIC PULP	AGGLUTINATION
1	Acquired hemolytic jaundice (lymphatic leucemia)	Frozen and thawed	+
		Distilled water	+
2	Acquired hemolytic jaundice (Boeck's sarcoid)	Distilled water	+
3	Acquired hemolytic jaundice (cause unknown)	Distilled water	+
4	Acquired hemolytic jaundice (cause unknown)	Frozen and thawed	+
5	Congenital hemolytic jaundice	Frozen and thawed	0
6	Congenital hemolytic jaundice	Frozen and thawed	0
7	Congenital hemolytic jaundice	Frozen and thawed	0
8	Thrombocytopenic purpura	Frozen and thawed	0
9	Thrombocytopenic purpura	Frozen and thawed	0
10	Thrombocytopenic purpura	Distilled water	0
11	Thrombocytopenic purpura	Distilled water	0
12	Congestive splenomegaly (Banti's syndrome)	Frozen and thawed	0
13	Congestive splenomegaly (Banti's syndrome)	Frozen and thawed	0
14	Congestive splenomegaly (Banti's syndrome)	Frozen and thawed	0
15	Gaucher's disease	Frozen and thawed	0
16	Gaucher's disease	Frozen and thawed	0

not agglutinated. Nor was agglutination observed when washed red blood cells alone or subsequent to incubation with the splenic pulp from patients with thrombocytopenic purpura, congestive splenomegaly (Banti's syndrome), or Gaucher's disease were exposed to the antihuman serum rabbit serum.

Inspection of the data in Table II shows that the washed red blood cells from the splenic pulp of three patients with acquired hemolytic jaundice were more strongly agglutinated by the antihuman serum rabbit serum than were

TABLE II. COMPARATIVE AGGLUTINATION TITERS OF ANTIHUMAN SERUM RABBIT SERUM (COOMBS' SERUM) AGAINST WASHED RED BLOOD CELLS FROM THE PERIPHERAL BLOOD AND FROM THE SPLENIC PULP OF PATIENTS WITH ACQUIRED HEMOLYTIC JAUNDICE; ALSO SHOWN ARE TITERS AGAINST WASHED NORMAL RED BLOOD CELLS SUBSEQUENT TO INCUBATION WITH THE SPLENIC PULP OF THESE PATIENTS

CASE	CELLS		RECIPROCALS OF SERUM DILUTIONS										
			0	2	4	8	16	32	64	128	256	512	1,024
1	Patient's cells	From peripheral blood	2+	1+	1+	0	0	0	0	0	0	0	0
		From splenic pulp	4+	3+	2+	2+	2+	1+	1+	1+	1+	1+	0
	Normal cells	After incubation with splenic pulp	1+	0	0	0	0	0	0	0	0	0	0
2	Patient's cells	From peripheral blood	2+	1+	1+	0	0	0	0	0	0	0	0
		From splenic pulp	3+	3+	3+	2+	2+	1+	0	0	0	0	0
	Normal cells	After incubation with splenic pulp	1+	0	0	0	0	0	0	0	0	0	0
3	Patient's cells	From peripheral blood	2+										
		From splenic pulp	4+										
	Normal cells	After incubation with splenic pulp	1+										

those derived from the peripheral blood of the same patients. Not shown in the tables is the fact that the red blood cells from the splenic pulp and peripheral blood of two additional patients with congenital hemolytic jaundice failed to agglutinate with the test serum. It was noted that the red blood cells from the splenic pulp of the patients with acquired hemolytic jaundice, when suspended in homologous normal serum, showed marked spontaneous agglutination but not when placed in physiologic salt solution. On the other hand, the red blood cells from the splenic pulp of two of the patients with thrombocytopenic purpura showed only rouleaux formation when suspended in homologous serum. When 25 per cent human albumin was used instead of the antihuman serum rabbit serum in performing any of the positive agglutination tests already described, it was invariably found to be less sensitive or to be incapable of causing any agglutination.

DISCUSSION

The results of the present experiments indicate that the spleen is a source of a substance which causes the washed red blood cells of certain patients with acquired hemolytic jaundice to be agglutinated by an immune serum developed in rabbits against human serum (Coombs' serum). Presumably this agglutination is due either to the adsorption of a substance on the surface of the red blood cell or to a modification of its surface structure by contact with an active substance in the splenic pulp. On the basis of current assumptions, the agglutination test may be in fact a test for the presence of adsorbed serum globulins. Indeed, the test originally was developed for the detection of blocking antibody on sensitized red blood cells. Even so, this does not necessarily allow the conclusion that the agglutinability of the red blood cells in acquired hemolytic jaundice is the result of an immunologic process in the strict sense, that is, that the patient's red blood cells, acting as an antigen, have in some way caused the development in his own serum of specific* antibody with avidity for the red blood cells. Although this is entirely possible, the seemingly nonspecific agglutination of red blood cells, for example by antiserum developed in rabbits against Type 14 pneumococcus[10] or by certain viruses[11] with which the animal has had no previous contact, is today too well known to allow so restricted an interpretation. Indeed, perhaps the association with a variety of infections and neoplastic conditions favors the interpretation of a nonspecific adsorption of a substance by the red blood cells.

The antiserum was developed by the injection of human serum into rabbits. The property of agglutinability of the normal red blood cells, however, was probably conferred by the splenic cells rather than by the presence of serum in the splenic pulp. In the first place, the patient's serum was largely removed from the splenic pulp by the numerous washings employed in the preparations made from the fresh spleens. Second, Shen and associates[12] have shown that even forty-eight hour incubation with the serum from one of these patients did

*In this paper the terms specific and nonspecific are used in order to refer to the reaction of an antibody and not to the formation of antibody. Thus an antibody formed in response to antigen A will react specifically with antigen A but may also react nonspecifically with substance B.

not cause normal red blood cells to become agglutinable by the antihuman serum rabbit serum. Third, the demonstration by Dougherty, Chase, and White[13] that rabbit lymphocytes contain a globulin identical with normal serum gamma globulin makes it reasonable to suppose that a similar relationship obtains between cells in the human splenic pulp and the human serum used as an antigen in the preparation of the test rabbit serum.

Other evidence that the spleen is a potent source of the substance causing agglutinability of the red blood cells is the fact already cited that splenectomy in some patients with acquired hemolytic jaundice causes a decrease in the agglutinability of the circulating red blood cells. This was the case in two of these patients. Moreover, it was shown here that the washed red blood cells of the patients with acquired hemolytic jaundice, when removed from the splenic pulp, were always more strongly agglutinated than were those from the general circulation. On the other hand, the red blood cells of two patients with congenital hemolytic jaundice, whether derived from the splenic pulp or from the peripheral blood, were not agglutinated by the test serum. This negative finding in congenital hemolytic jaundice is particularly significant because there is ample evidence that in this disease the spheroidal red blood cells are selectively retained and sequestered in the splenic pulp.[14, 15] Consequently, if a substance with affinity for the red blood cells was present in this disease, ample opportunity must have existed for its action on the red blood cells in the splenic pulp to become apparent. At any rate it is clear that in these cases of acquired hemolytic jaundice in which the patients' circulating red blood cells, and especially those in the splenic pulp, exhibited such a property, the spleen possessed a special capacity for causing normal red blood cells also to become agglutinable by the antihuman serum rabbit serum. In view of the short duration (two hours) of the crude exposure of the normal red blood cells to the splenic pulp in vitro compared with the prolonged opportunity for contact by the patients' red blood cells in vivo, it is not surprising that the agglutination of the normal red blood cells was not more pronounced.

The red blood cells in the peripheral blood of the majority of the twelve patients with acquired hemolytic jaundice and a positive Coombs test that have been studied by us have displayed significantly increased mechanical fragility and, in some instances, increased osmotic fragility as well.[12] These findings indicate that the red blood cells have been damaged, and therefore when mechanically fragile are at least unusually susceptible to destruction by the motion of the circulation. The observation that the red blood cells from the splenic pulp exhibited marked agglutination in homologous normal serum is probably the result of the presence of the adsorbed substance that under other circumstances caused the positive Coombs test. This spontaneous agglutinability of the red blood cells in the splenic pulp may be suspected as the basis of the characteristic histologic picture of congestion and infarction in the spleen that has been reported, according to Dameshek and Schwartz,[4] by several authors. It is thus possible that the passive adsorption by the red blood cells of a substance produced in the splenic pulp (and probably in other organs) may result in increasing local stagnation and injury to the red blood cells. The course of

the resulting acquired hemolytic anemia may sometimes be favorably altered by interrupting this vicious cycle by removal of the spleen. On the other hand, in other patients with a positive Coombs test splenectomy may not alter the titer or diminish the anemia, presumably because other organs share this function of the spleen.

SUMMARY AND CONCLUSIONS

Incubation of normal red blood cells of compatible blood group with the splenic pulp of four patients with acquired hemolytic jaundice caused these cells to become agglutinable by an undiluted immune serum developed in rabbits against human serum (Coombs' serum). When similar experiments were conducted using spleens from three patients with congenital hemolytic jaundice, three with thrombocytotopenic purpura, three with congestive splenomegaly (Banti's syndrome), and two with Gaucher's disease, the results were negative.

Washed red blood cells removed from the splenic pulp of three of the patients with acquired hemolytic jaundice were more strongly agglutinated by the Coombs serum than were those derived from the peripheral blood. Similar experiments made with washed red blood cells derived from the splenic pulp or peripheral blood of two patients with congenital hemolytic jaundice resulted in no agglutination.

Red blood cells removed from the splenic pulp of three patients with acquired hemolytic jaundice exhibited marked spontaneous agglutination in homologous normal serum but not in physiologic salt solution. Similar experiments with red blood cells removed from the splenic pulp of two patients with thrombocytopenic purpura yielded negative results.

It is concluded that the spleen in certain patients with acquired hemolytic jaundice is a source of a substance responsible for the agglutination of the patient's red blood cells by antihuman serum rabbit serum. That the agglutination is probably the result of a reaction between a substance on the red blood cells and an antibody in the rabbit serum developed against human serum neither proves nor disproves that the substance on the red blood cells was elaborated by an immunologic reaction.

It is concluded that the spontaneous agglutination of the patient's red blood cells, especially when derived from the spleen, and the characteristic congestion and infarction of that organ observed by others suggest that in these instances of acquired hemolytic jaundice spontaneous agglutination of the red blood cells is a factor either causing or resulting from stagnation of these cells in the spleen. The resulting injury to the red blood cells is manifest in those that escape from their temporary sequestration in the spleen by the increased osmotic and mechanical fragilities of the circulating red blood cells of the majority of these patients. If not destroyed in the spleen, when mechanically fragile, such red blood cells may well be abnormally susceptible to the trauma of the motion of the circulation.

We are greatly indebted to Dr. Louis K. Diamond and to Dr. B. Harrison Ragle for an opportunity to carry out observations on spleens removed from their patients at operation.

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DEMONSTRATION OF HETEROPHILE ANTIBODIES IN THE CEREBROSPINAL FLUID FROM PATIENTS WITH INFECTIOUS MONONUCLEOSIS

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PAUL and Bunnell[1] in 1932 demonstrated a consistent and marked increase of heterophile antibodies in the serum of patients with infectious mononucleosis. Subsequently it was shown[2, 3, 4] that by absorption methods these sheep hemagglutinins could be differentiated from those present in the sera of normal individuals and in serum diseases. In 1931 Epstein and Dameshek[5] described a patient with meningo-encephalitis in the course of infectious mononucleosis. Since then there have been many reports of nervous system involvement with this disease.[6]

Several unsuccessful attempts have been made to demonstrate heterophile antibodies in the cerebrospinal fluid of patients with infectious mononucleosis.[7-10] It has been emphasized[11, 12] that there are relatively small amounts of antibodies in the cerebrospinal fluid as compared with those in circulating blood. The purpose of this investigation was to demonstrate heterophile antibodies in the cerebrospinal fluid of patients with infectious mononucleosis by employing large volumes of spinal fluid and dilute sheep erythrocyte suspensions as antigen.

MATERIALS AND METHODS

A 1 per cent washed sheep erythrocyte suspension was prepared and 0.1 ml. of it was added to 1 ml. and 0.5 ml. of fresh spinal fluid in Kahn-sized test tubes and shaken. Saline controls were prepared. The tubes were centrifuged for five minutes at 2,500 revolutions per minute and macroscopic agglutination was demonstrated upon resuspending the cells by shaking. Results were recorded from negative to 4 plus, depending upon the degree of agglutination.

A modification of Davidsohn's[13] absorption method was adopted to study the heterophilic nature of the agglutinins found in cerebrospinal fluid. One milliliter of spinal fluid was placed in Kahn-sized test tubes. To one was added 0.5 ml. of guinea pig kidney antigen and to the other 0.5 ml. of boiled beef erythrocytes. After incubation for ten minutes at 37° C. these were centrifuged at 2,500 revolutions per minute for ten minutes. One milliliter of the supernatant fluid of each was then treated in the manner described previously.

EXPERIMENTAL RESULTS

The blood and spinal fluid of six patients with infectious mononucleosis were examined for heterophile antibodies. (Table I.) Lumbar punctures were done the same day or the day following the demonstration of a blood heterophile

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TABLE I. A COMPARISON OF THE BLOOD AND CEREBROSPINAL FLUID HETEROPHILE ANTIBODY TITERS

PATIENT	DIAGNOSIS	BLOOD: HETEROPHILE ANTIBODY TITER (DAVIDSOHN METHOD)			CEREBROSPINAL FLUID: HETEROPHILE ANTIBODY TITER (CENTRIFUGE METHOD)				CELL COUNT	TOTAL PROTEIN (MG./100 ML.)
		UNABSORBED	ABSORBED		UNABSORBED		ABSORBED			
			G.P.K.	B.B.E.	1 ML.	0.5 ML.	G.P.K.	B.B.E.		
A	Infectious mononucleosis	1:3,584	1:1,796	Neg.	2+	±	1+	Neg.	4	16
B	Infectious mononucleosis	1:112	1:56	Neg.	1+	Neg.	Qns.	Qns.	15*	77
C	Infectious mononucleosis	1:448	1:224	Neg.	4+	3+	2+	Neg.	20†	13
D	Infectious mononucleosis	1:1,792	1:896	Neg.	3+	2+	2+	Neg.	4	34
E	Infectious mononucleosis	1:896	1:448	Neg.	2+	Qns.	1+	Neg.	4	42
F	Infectious mononucleosis	1:3,584	1:1,796	Neg.	4+	2+	3+	Neg.	2	15
G	Tuberculous meningitis	Neg.			±	Neg.	Neg.	Neg.	108	536
H	Subarachnoid hemorrhage	1:56	Neg.	1:28	2+	Neg.	Neg.	2+	90	110
I	Trichinosis	1:112	1:56	Neg.	3+	2+	2+	Neg.	3	40

*Neutrophiles 6 per cent, lymphocytes 94 per cent.
†Lymphocytes 100 per cent.
G.P.K., guinea pig kidney antigen.
B.B.E., boiled beef erythrocytes.
Qns., quantity not sufficient.

antibody titer. The serum titers ranged from 1:112 through 1:3,584 with Davidsohn's method, and the specificity was verified by his absorption procedure. A parallel series using similar dilutions of serum and the method employed in testing spinal fluids resulted in approximately an eightfold increase in these titers as compared with the usual technique of serum titration. Hemagglutinins, from 1 plus to 4 plus, were present in the spinal fluids of all these patients and they had the same absorption pattern as those found in the blood. The sera and spinal fluids of these patients were negative for Kahn and Kolmer-Wassermann tests. The spinal fluid cell counts were increased in Patients B and C and lymphocytes predominated. No growth was obtained upon culture. The colloidal gold reactions were normal with the exception of Patient B in whom a slight mid-zone curve resulted, and this may have been due to the elevated total protein.

In this study 654 routine spinal fluids were examined and nine had demonstrable sheep hemagglutinins. Six of these were from the patients with infectious mononucleosis described previously. The remaining three (0.4 per cent) so-called false positive hemagglutinations included a case of tuberculous meningitis (cerebrospinal fluid elevated protein), one of subarachnoid hemorrhage

(passive transfer of agglutinins), and one of trichinosis. Although the heterophile antibodies in the first two were absorbed with guinea pig kidney extract, the hemagglutinins in the third persisted after this absorption. The heterophile agglutinins in this patient with trichinosis remain unexplained, although the possibility of concomitant infectious mononucleosis exists.

DISCUSSION

A linear relationship does not appear to exist between the blood and cerebrospinal fluid heterophile antibody titers in infectious mononucleosis during the acute phase of illness. However in both the cerebrospinal fluid and blood, the sheep cell agglutinins persisted following guinea pig kidney antigen absorption and were absorbed with boiled beef erythrocytes. (Table I.) In a preliminary series of experiments the same spinal fluids were tested with human red cells sensitized with the virus of Newcastle disease,[14] and a high degree of nonspecificity was found. Encephalitis associated with infectious mononucleosis as suggested by Tidy[15] was noted only in Patient D whose cerebrospinal fluid hemagglutinins were not significantly higher than the others.

SUMMARY

A simple method is described for the demonstration of heterophile agglutinins in cerebrospinal fluid. Of 654 spinal fluids tested, six from patients with infectious mononucleosis had heterophile antibodies in both blood and cerebrospinal fluid and one of these showed nervous system involvement.

The authors wish to express their appreciation to Dr. Morris L. Rakieten for advice and for conducting the experiments with the virus of Newcastle disease.

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THE USE OF YEAST PHASE ANTIGENS IN A COMPLEMENT FIXATION TEST FOR HISTOPLASMOSIS

II. Results With Ground Antigens

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A COMPLEMENT fixation test for the detection of antibodies against *Histoplasma capsulatum* employing the yeast phase of the organism as antigen has been described previously from this laboratory.[1] In the presence of yeast phase antigens, immune rabbit sera fixed complement in high dilutions, but immunologic cross reactions with *Blastomyces dermatitidis* described by others[2, 3, 4] working with mycelial antigens were noted also in our studies.

Yeast phase antigens described in the earlier report were completely satisfactory for short periods but tended to develop anticomplementary activity after storage for several weeks at 3 to 6° C. Also since in our complement fixation test the per cent hemolysis was determined by comparison with centrifugalized standards, it was occasionally difficult, in the range nearing complete hemolysis, to differentiate nonhemolyzed sensitized sheep's cells from the residual sediment of turbid antigens. These minor difficulties instigated the search for an antigen which would meet more adequately the precise end points and demands of the test. This report describes the use of ground yeast phase antigens.

MATERIALS AND METHODS

Antigens.—Antigens were prepared from the yeast phase of each of three strains of *H. capsulatum* (G-2, G-5, G-6*) and two strains of *B. dermatitidis* (A-1 and A-5†). The yeast phase organisms were obtained from cultures grown at 37° C. on glucose cystine agar[5] containing 35 units of penicillin and 40 units of streptomycin per milliliter of medium. After the fifth day of incubation the growth was washed gently with sterile buffered saline from the surface of at least five slants, filtered through sterile gauze, and centrifugalized. The sediment was resuspended in 50 ml. of sterile buffered saline containing Merthiolate (1:10,000 final concentration) and stored at 37° C. Organisms thus treated were usually not viable after seventy-two hours, but since further incubation did not alter the resultant antigen, exposure to Merthiolate was continued as a precautionary measure for one week.

The Merthiolate-killed suspension was centrifugalized for thirty minutes at 3,000 revolutions per minute, the supernate discarded, and the sediment transferred to a small TenBroeck glass tissue grinder. The organisms were then ground by hand for thirty minutes. One to three milliliters of sterile buffered saline were added at five-minute intervals during grinding. Finally the ground antigen mixture was centrifugalized until the supernate was completely free of organisms. This clear saline supernate constituted the antigen. The grinding procedure usually was repeated several times with each sediment and the supernates were saved

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*The G-2, G-5, and G-6 strains of *H. capsulatum* were reverted from the mycelial phase of strains 715, 952, and 650 respectively, from the collection of Dr. N. F. Conant at Duke University, Durham, N. C.

†A-1 strain of *B. dermatitidis* and A-5 (Duke 930) are isolates from human cases received from Dr. Conant at Duke University.

in separate containers. Supernates showing adequate and comparable titers were pooled, while those of reduced or unsatisfactory potency were discarded. After the addition of Merthiolate 1:10,000 as preservative, the pooled material was titrated and used as the stock antigen.

Antisera, diluent, sheep red blood cells, hemolysin, and complement were prepared exactly as described in the previous report[1] and the complement fixation test of Kent and Rein[6] was again employed.

RESULTS

Experiment 1.—The optimal dilution of each lot of antigen was determined by titration. The antigenic unit was established as being the greatest concentration contained in 0.2 ml. beyond which further increase failed to enhance the serum reaction. It was required that this antigen concentration be neither anticomplementary nor hemolytic. The optimal dilutions of the ground antigen

TABLE I. DETERMINATION OF THE OPTIMAL DILUTION OF GROUND ANTIGEN USING THE G-2 STRAIN OF H. CAPSULATUM AND SERUM PREPARED IN RABBITS AGAINST G-2

DILUTION OF ANTIGEN (0.2 ML.)	POSITIVE SERUM DILUTIONS IN (0.2 ML.)						CONTROLS		
	1:10	1:20	1:40	1:80	1:160	1:320	ANTIGEN	COMPLEMENT	CELLS
1:1	0%	0%	0%	0%	0%	0%	90%	100%	0%
1:1.3	0	0	0	0	10	50	AC		
1:2*	0	0	0	0	20	100	100		
1:2.6	0	0	0	0	30	100	100		
1:4	0	0	0	30	80	100	100		
1:5.3	0	0	20	60	95	100	100		
1:8	5	20	50	95	100	100	100		

0, No hemolysis; 100, complete hemolysis; AC, almost complete hemolysis.
*Optimal dilution was 1:2 since there was good fixation but no trace of anticomplementary activity.

varied between 1:2 to 1:4, and Table I shows a typical titration in which the G-2 antigen was shown to have an optimal dilution of 1:2. As can be seen in Table II, the ground antigens prepared by the described method have been used in this laboratory for twelve consecutive weeks with no development of anticomplementary activity and with no appreciable loss in titer when stored in the refrigerator.

TABLE II. DETERMINATION OF THE STABILITY OF G-5 GROUND ANTIGEN WHEN USED AT OPTIMAL DILUTION OVER A TWELVE-WEEK STORAGE PERIOD AT 3 TO 6° C.

STORAGE AT 3-6° C. (WK.)	G-5 SERUM DILUTIONS					CONTROLS		
	1:10	1:20	1:40	1:80	1:160	ANTIGEN	COMPLEMENT	CELLS
1	0%	0%	0%	0%	20%	100%	100%	0%
2	0	0	0	0	15	100	100	0
3	0	0	0	0	15	100	100	0
6	0	0	0	0	40	100	100	0
12	0	0	0	0	20	100	100	0

0, Hemolysis; 100, complete hemolysis.

Experiment 2.—The results obtained with yeast phase ground antigens are demonstrated in Tables III and IV. Fixation of complement was obtained in similar titers with homologous and heterologous strains of histoplasma antigens

TABLE III. REACTIONS IN THE COMPLEMENT FIXATION TEST EMPLOYING AN OPTIMAL DILUTION OF GROUND ANTIGEN FROM THE G-2 STRAIN OF H. CAPSULATUM AND THE SERA OF RABBITS INOCULATED WITH THE G-2, G-5, AND G-6 STRAINS OF H. CAPSULATUM, THE A-1 AND A-5 STRAINS OF B. DERMATITIDIS, AND B. BRASILIENSIS, S. SCHENCKII, C. ALBICANS AND NORMAL CONTROL RABBIT SERUM

	SERUM DILUTIONS							CONTROLS		
SERA	1:5	1:10	1:20	1:40	1:80	1:160	1:320	ANTIGEN	COMPLEMENT	CELLS
G2	0%	0%	0%	0%	0%	15%	50%	100%	100%	0%
G-5	0	0	0	0	5	60	100			
G-6	0	0	0	5	50	95	100			
A-1	0	0	20	40	95	AC	100			
A-5	0	15	50	90	100	100	100			
B. brasiliensis	100	100	100	100	100	100	100			
S. schenckii	95	100	100	100	100	100	100			
C. albicans	100	100	100	100	100	100	100			
Normal	100	100	100	100	100	100	100			

0, No hemolysis (complete fixation); 100, complete hemolysis (no fixation); AC, almost complete hemolysis.

The titer was taken as being the highest dilution of serum showing 50 per cent or less of hemolysis.

and antisera. For example, when the G-2 strain of *H. capsulatum* was used as the antigen source (Table III), the G-2, G-5, and G-6 rabbit antisera gave titers of 1:320, 1:80, and 1:80 respectively. With the G-5 antigen (Table IV), the G-2, G-5, and G-6 antisera yielded titers of 1:320, 1:160, and 1:160 respectively.

Sera prepared against *Candida albicans* (E-11*), *Sporotrichum schenckii* (F-20*), and *Blastomyces brasiliensis* (B-3†) as well as normal rabbit sera did not fix complement in the presence of histoplasma antigens. On the other hand,

TABLE IV. REACTIONS IN THE COMPLEMENT FIXATION TEST EMPLOYING AN OPTIMAL DILUTION OF THE G-5 STRAIN OF H. CAPSULATUM AS ANTIGEN AND THE SAME SERA USED IN TABLE III

	SERUM DILUTIONS							CONTROLS		
SERA	1:5	1:10	1:20	1:40	1:80	1:160	1:320	ANTIGEN	COMPLEMENT	CELLS
G-2	0%	0%	0%	0%	0%	25%	50%	100%	100%	0%
G-5	0	0	0	0	0	15	70			
G-6	0	0	0	0	10	50	100			
A-1	0	0	20	40	95	100	100			
A-5	0	0	0	10	80	100	100			
B. brasiliensis	100	100	100	100	100	100	100			
S. schenckii	100	100	100	100	100	100	100			
C. albicans	100	100	100	100	100	100	100			
Normal	100	100	100	100	100	100	100			

See footnotes to Table III.

the A-1 and A-5 *B. dermatitidis* antisera fixed complement in dilutions ranging between 1:20 to 1:40 (Tables III and IV). This cross reaction in lower dilutions between the histoplasma antigen and blastomyces antisera was likewise noted and discussed in our previous report.[1]

Experiment 3.—In order to evaluate further the results obtained in Experiment 2 wherein both histoplasma and blastomyces antisera fixed complement in the presence of the histoplasma antigen, ground antigens of the A-5 strain

*Isolated from patient material at the Army Medical Center.

†Strain from human material received from Dr. Conant.

TABLE V. CROSS REACTIONS IN THE COMPLEMENT FIXATION TEST EMPLOYING THE A-5 STRAIN OF B. DERMATITIDIS AS ANTIGEN AND SERA OF RABBITS INOCULATED WITH THE A-1 AND A-5 BLASTOMYCES ANTIGENS AND THE G-2, G-5, AND G-6 HISTOPLASMA ANTIGENS

	SERUM DILUTIONS							CONTROLS		
SERA	1:5	1:10	1:20	1:40	1:80	1:160	1:320	ANTIGEN	COMPLEMENT	CELLS
A-1	0%	0%	0%	30%	100%	100%	100%	100%	100%	0%
A-5	0	0	0	10	80	AC	100			
G-2	0	0	0	0	50	95	100			
G-5	0	0	0	0	3	20	100			
G-6	0	0	0	30	65	AC	100			
B. brasiliensis	95	100	100	100	100	100	100			
S. schenckii	95	100	100	100	100	100	100			
C. albicans	AC	100	100	100	100	100	100			
Normal	100	100	100	100	100	100	100			

See footnotes to Table III.

of *B. dermatitidis* were employed against the same antisera. In the presence of the A-5 antigen both the A-1 and A-5 rabbit antisera fixed complement in dilutions of 1:40 (Table V). The G-2, G-5, and G-6 histoplasma antisera fixed complement in dilutions of 1:80, 1:160, and 1:40 respectively. With the exception of the G-5 antisera, the titers of the histoplasma antisera were lower in the presence of the blastomyces antigen than in the presence of their specific antigens (see Tables III and IV). The significance of these results will be discussed below.

DISCUSSION

The preparation of ground yeast phase antigens of *H. capsulatum* and *B. dermatitidis* for use in the complement fixation test is herein described. Rabbit antisera prepared from homologous and heterologous strains of *H. capsulatum* and *B. dermatitidis* fixed complement in the presence of both histoplasma and blastomyces antigens, while sera prepared against *C. albicans*, *S. schenckii*, and *B. brasiliensis* as well as normal rabbit sera did not.

The cross reaction pattern noted when the whole yeast phase organisms of *H. capsulatum* and *B. dermatitidis* were used[1] was even more pronounced with the use of ground yeast phase antigens. The A-1 and A-5 blastomyces antisera having specific titers of 1:40 fixed complement in the presence of ground histoplasma antigens in serum dilutions of 1:20 to 1:40. (With whole organisms this cross had been found only in dilutions of 1:10 or less.[1]) Evidence that a strong antigenic relationship exists between these two organisms was even more apparent when histoplasma antisera was tested for complement-fixing antibodies in the presence of blastomyces ground antigens. Histoplasma sera having specific titers of 1:160 to 1:320 reacted with blastomyces antigens in serum dilutions ranging from 1:40 to 1:160. However, the antiserum against the G-5 strain of *H. capsulatum* fixed complement to the same dilution in the presence of both the specific and *B. dermatitidis* antigens, while the G-2 antiserum with a specific titer of 1:320 fixed complement in the presence of blastomyces antigen only to a dilution of 1:80. This suggests that the common antigenic component of the two organisms may appear in varying degrees in different strains and emphasizes the risk involved in evaluating the results of complement fixation

tests for histoplasmosis which utilize only the antigen of *H. capsulatum*. On the other hand, the use of both *H. capsulatum* and *B. dermatitidis* antigens allows for a more comprehensive and critical analysis of the test proper and of the results obtained therein.

The ultimate value of the complement fixation test as a potential diagnostic aid in the diagnosis of histoplasmosis in patient material is yet to be determined. Whereas the tests now in use employ histoplasmin, a filtrate of the mycelial form of *H. capsulatum*, as antigen, it is our belief at this time that tests aimed toward the detection of antibodies against *H. capsulatum* as it appears in pathologic processes, that is the yeast phase, should be investigated more fully. Immunologic cross reactions occur between blastomyces and histoplasma rabbit antisera regardless of the phase employed as antigen, but in our experience the use of histoplasmin as antigen has not been as satisfactory as yeast phase antigens because of its high incidence of anticomplementary activity and the low serum titers obtained.[7]

The use of whole yeast phase organisms as antigens is relatively simple and has been described earlier by us.[1] Ground antigens offer two advantages: (1) a clearer, more readily readable test, and (2) greater stability of the antigen. A disadvantage (if it may be called such) of the ground antigen is the greater degree of cross reaction between *H. capsulatum* and *B. dermatitidis*. This, however, as mentioned, can usually be better evaluated by the judicious use of antigens prepared from both organisms.

SUMMARY

The preparation of ground antigens from the yeast phase of *H. capsulatum* and *B. dermatitidis* for use in complement fixation is described.

Ground yeast phase antigens are as specific as antigens made from whole organisms; they give more clear-cut end points and do not develop anticomplementary activity during storage at 3 to 6° C.

The immunologic relationship between *H. capsulatum* and *B. dermatitidis* noted in a previous report concerning yeast phase antigens has been confirmed further.

The use of both *H. capsulatum* and *B. dermatitidis* antigens in evaluating complement-fixing antibody responses to *H. capsulatum* is recommended.

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ANTIGENIC DIFFERENCES AMONG INFLUENZA A VIRUSES, INCLUDING SEROLOGIC RESPONSE OF PATIENTS

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THE failure of influenza virus vaccines to protect immunized individuals during the influenza outbreak that occurred early in 1947 and the demonstration of antigenic differences between the strains of virus contained in the vaccines and those responsible for the epidemic[1-3] have aroused further interest in the problem of strain variations among influenza viruses.[1-5] Strains of influenza virus were also isolated during the same epidemic when it occurred in Boston, and they too showed sharp differences from the classic PR8 strain of influenza A. The present report presents the results of studies on (1) the antigenic relationships of these recently isolated strains to several that were obtained in a local outbreak in 1943-1944[6] and to several other well-known strains of influenza viruses and (2) the immunologic response of patients to their homologous virus and to other strains of influenza virus.

MATERIALS AND METHODS

Throat Washings.—Garglings were obtained on the first to the third day of the disease from thirteen patients who were acutely ill with clinical influenza and had temperatures of 100° F. or higher at the time. The washings were made with infusion broth containing 10 per cent horse serum and were collected between March 18 and April 17, 1947. Most of them were tested for virus immediately and then stored in sealed glass ampules in the dry ice cabinet. Garglings were also obtained from the patient P. W., an isolated instance of clinical influenza, on Dec. 6, 1947; the data on this patient are included in the tables, but will be discussed separately.

Virus Isolation.—Attempts were made to isolate influenza viruses from the throat washings, and in two instances from nasal secretions, by inoculation of embryonated eggs by both the amniotic and allontoic routes as previously described.[7] Once the virus was established, subsequent passages all were made in eggs by the allantoic route.

Serologic Tests.—The methods used in the hemagglutination inhibition and complement fixation tests were similar to those used in previous studies.[6] Serum neutralization tests were carried out in chick embryos by two different methods: one of these employed the allantoic route as described by Hirst,[8] and the other was a modification in which 0.25 ml. amounts of the serum-virus mixtures were injected into the yolk sac of 7-day-old eggs which were then incubated for five days at 35° C.; the deaths were recorded and the serum protection titers based on the LD_{50} were calculated.[9] The latter method eliminated the necessity of opening each egg individually to test for the presence of influenza virus.

Acute (third day or earlier) and convalescent (tenth day or later) phase serum specimens were obtained from the patients and stored at –20° C. in rubber-stoppered tubes. Rabbit antiserums for the study of antigenic differences were prepared against each virus. Albino rabbits, weighing 2.5 to 3.5 kilograms, were first bled to obtain control serum specimens; they were then injected intravenously with 0.1 ml. of virus-infected allantoic fluid and bled again after ten to fourteen days. The serums were heated at 60° C. for twenty

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minutes and tested for antibody by the hemagglutination inhibition method. If a satisfactory rise in titer was obtained, the rabbits were bled by cardiac puncture and the serums stored in sealed glass tubes at -20° C. until used.

RESULTS

Virus Isolations.—Strains of influenza virus were obtained from the throat washings of three patients (E. B., G. P., and D. W.) of the thirteen from whom washings were obtained during the March-April, 1947, outbreak, and a strain also was obtained from the nasal secretions of one of them (E. B.). In each instance the virus was first identified on the second amniotic passage in embryonated eggs and was detected in both amniotic and allantoic fluid at that time.* The throat washings of E. B. also yielded the virus after four allantoic passages. Each of the remaining throat washings and the other sample of nasal secretions were passed blindly four or five times both by the amniotic and by the allantoic routes without yielding any detectable virus. Two throat washings which had given negative results following passages in embryonated eggs were inoculated intranasally in mice and lung suspensions were passed, three times in one instance and six times in the other, without producing visible lesions.

Thus, influenza viruses were isolated only with difficulty during this outbreak, and the amniotic route of inoculation gave the best results. Similar difficulty in establishing viruses in eggs was also reported by others.[1]

Serologic Tests in Patients' Serums.—The results of the hemagglutination inhibition and complement fixation tests on the serums of twenty patients who were acutely ill with clinical influenza between the middle of March and the middle of April, 1947, and in the case of P. W. who was ill in December of that year are shown in Table I. It is seen, first of all, that no significant rises in titer were obtained with the Lee strain of influenza B in any instance. With the other viruses used, both the absolute titers and the extent of the rises in titer varied in the same patient with the different viruses, and in a number of instances there were similar discrepancies in the titers obtained with the same virus by the two tests. Some of the latter discrepancies were elicited even when the two tests were done simultaneously with the same serum dilutions and the same virus antigens.

A fourfold or greater rise in titer was obtained by one or both tests with the PR8 strain or with one of the epidemic strains in each of the twenty patients who were ill in the spring. In five of these patients such rises were elicited either by only one of the virus antigens or by only one of the tests, but not with the other test employing the same antigen nor by either test with any of the other virus strains. The serums of one of these patients, D. W., showed a significant rise in titer only by the agglutination inhibition test with one of the epidemic strains, GP, but showed either no change or only a twofold increase in titer with the other viruses used, including the strain isolated from the patient's own throat washings.

The tests with the PR8 strain yielded the greatest proportion of positive results. Only one patient, J. M., showed no increase at all by the agglutination

*For subsequent serologic tests with rabbit antiserums and with patients' serums the viruses were used only after they had been passed through eighteen to twenty additional allantoic passages.

TABLE I. RESULTS OF SEROLOGIC TESTS IN PATIENTS

PATIENT	VIRUS ISOLATION*	VIRUS ANTIGEN											
		PR8		SWINE		EB		GP		DW		LEE	
		H. I.	C. F.	H. I.	C. F.	H. I.	C. F.	H. I.	C. F.	H. I.	C. F.	H. I.	C. F.
E. B.	+	8/64†	32/512	<4/8	16/128	32/128	–/512	<4/16	–/256	<4/8	–/256	16/16	8/8
G. P.	+	4/16	<4/64	4/16	8/32	32/64	4/64	8/32	<4/64	4/4	<4/64	4/4	8/8
D. W.	+	4/8	32/64	8/8	32/64	32/32	64/128	<4/16	64/128	<4/<4	32/64	16/16	32/32
M. C.	0	8/32	8/32	<4/4	8/16	64/64	16/64	16/16	32/64	<4/4	16/32	4/4	32/16
M. F.	0	4/32	8/64	8/64	16/64	128/128	16/64	8/16	16/128	<4/4	8/64	8/8	128/128
A. A.	0	64/512	16/128	512/2048	32/64	64/256	16/64	4/16	32/128	8/128	16/64	8/8	8/8
C. H.	0	64/128	8/32	64/256	8/32	128/128	16/64	8/32	32/128	<4/8	16/32	32/32	16/16
M. P.	0	128/256	32/64	128/256	16/32	128/512	8/64	16/64	32/64	16/16	32/64	128/128	8/8
J. M.	0	32/32	----	64/64	----	64/32	8/32	64/32	64/256	<4/<4	64/128	64/64	64/64
O. B.	0	8/64	32/256	16/16	AC	128/4‡	AC	8/4‡	AC	<4/<4	AC	32/32	32/32
D. N.	0	32/1024	----	----	----	64/128	8/128	----	----	----	----	32/64	----
M. E.	–	32/64	32/128	16/64	32/256	64/128	16/128	16/8	16/256	<4/4	16/128	<4/<4	32/32
J. J.	–	64/512	4/512	64/256	----	128/512	16/256	256/256	16/64	8/16	16/32	8/16	<4/<4
S. A.	–	8/32	<4/64	----	----	128/64	8/64	16/64	16/64	<4/4	4/64	32/32	16/16
G. R.	–	16/128	----	----	----	<4/4	16/16	<4/<4	32/32	<4/<4	8/8	8/8	----
V. B.	–	32/128	<4/32	----	----	32/128	16/64	<4/64	32/128	<4/16	8/32	8/8	<4/<4
M. K.	–	256/4096	32/512	512/4096	----	16/512	32/512	<4/512	----	<4/256	----	16/32	16/32
J. W.	–	16/256	4/256	<4/64	----	<4/128	----	<4/64	----	<4/16	----	64/128	16/16
H. B.	–	16/1024	<4/128	----	----	----	----	----	----	----	----	16/16	<4/<4
M. M.	–	8/32	8/8	----	----	----	----	----	----	----	----	64/64	32/32
P. W.§	+	8/8	4/4	----	16/16	----	8/8	----	----	----	----	8/8	----

H. I., Hemagglutination inhibition; C. F., complement fixation; AC, anticomplementary.

*+, Virus isolated; 0, attempts to isolate virus failed, ----, not attempted.

†Reciprocals of dilution end point: acute/convalescent (----, not done).

‡Same result three times.

§Agglutinin inhibition with own virus, 8/8.

inhibition test with this strain, and the complement fixation test was not done with this patient's serums. In this case a fourfold rise in titer was elicited only by the complement fixation test with one of the epidemic strains, EB. Two patients, D. W. and M. P., yielded only a twofold rise in titer by both tests with the PR8 strain; the latter showed a fourfold rise with two of the epidemic strains. Three other patients yielded a fourfold rise by one test with PR8 and a twofold rise by the other test with the same strain. The serums of all of the remaining patients showed a fourfold or greater rise in titer by both tests with the PR8 strain, and the increases in titer were greater with this strain than with any of the others used.

Significant rises in titer were less frequent and less striking with the DW strain than with the other two epidemic strains. With each of the epidemic strains, and particularly with the EB strain, the complement fixation test yielded an appreciably higher proportion of significant rises in titer than did the agglutination inhibition tests. The rises in titer with the Swine strain were less striking than those obtained with the PR8 or with the epidemic strains.

Antigenic Relationships Among Strains of Influenza Virus.—Because of the demonstration in a previous study[7] that the virus neutralization test is more sensitive than either the hemagglutination inhibition or the complement fixation test for the detection of strain differences, the neutralization test with rabbit antiserum was used to study the antigenic relationships of the strains isolated from this outbreak and a number of other strains. The method of Hirst[8] was used. The following strains of influenza virus were compared: EB, GP, and DW, isolated during the outbreak of March-April, 1947, in Boston; FM_1, isolated during the epidemic of the same season at Fort Monmouth, N. J.[2]; MA, MB, and CP, isolated during the 1943-1944 outbreak in Boston; PW, isolated from a sporadic case in Boston in December, 1947; and also the well-known PR8, Weiss, Swine, and Lee strains. The results are shown in Table II.

TABLE II. RESULTS OF IN OVO NEUTRALIZATION TESTS WITH INFLUENZA VIRUSES AND RABBIT ANTISERA USING THE ALLANTOIC ROUTE OF INFECTION

VIRUS		IMMUNE RABBIT SERUM PREPARED AGAINST										
STRAIN*	DOSE†	EB	GP	DW	FM_1	MA	MB	CP	PR8	WEISS	SWINE	LEE
EB	500	**594**‡	514	32	147	<4	<4	8	<4	<4	<4	<4
GP	500	182	**590**	6	128	—	—	—	<4	<4	<4	<4
DW	500	27	6	**113**	<16	—	—	—	<4	<4	<4	<4
FM_1	2,825	128	446	40	**152**	4	16	4	<4	<4	<4	<4
MA	5,000	<4	<4	<4	<4	**8**	41	<4	32	8	<4	<4
MB	158	4	<4	10	<4	32	**2048**	8	256	40	<4	—
CP	283	<4	<4	<4	<4	10	64	**512**	>4096	32	<4	<4
PR8	250	<4	<4	<4	<4	<4	64	512	**2048**	<4	<4	<4
Weiss	1,580	<4	<4	11	<4	—	512	—	128	**128**	<4	<4
Swine	1,580	<4	<4	<4	<4	<4	<4	<4	8	<4	**11**	<4
Lee	50	<4	<4	<4	<4	—	—	—	<4	<4	<4	**256**
PW§	200	<4	—	<4	<4	<4	<4	—	<4	<4	<4	<4

—, Not done.

*Strains MA, MB, and CP correspond to Case Numbers 68, 4, and 67 respectively in the report of the 1943-1944 epidemic.[6]

†Number of 50 per cent infective doses (ID_{50}) inoculated.

‡Reciprocal of 50 per cent protective titer of serum; homologous titer in bold type.

§Titer of homologous rabbit antiserum, 512.

None of the control rabbit serums showed any neutralization with any of the serums used, even in the lowest dilution tested, namely 1:4. In the neutralization tests with the immune rabbit serums, the three strains EB, GP, and DW showed sharp differences from the classic PR8, Weiss, and Swine strains of influenza A in that there was little or no cross neutralization. Furthermore, the DW strain was distinctly different in its reaction from the related EB and GP strains, although all three of these viruses were isolated from members of the staff of the Boston City Hospital during the same outbreak. The two latter strains appear to be very closely related to the FM_1 strain isolated at Fort Monmouth during the same season.

The influenza virus strains of the 1947 epidemic showed little or no relationship to the three strains isolated in the 1943-1944 outbreak in Boston. Of the three strains isolated from the latter outbreak, MA shows distinct differences from the classic PR8, Weiss, and Swine strains; MB is related to the PR8 strain, and CP is almost indistinguishable from PR8 in these neutralization tests. Furthermore, strain MA is clearly differentiated from the MB and CP strains; the two latter strains show a considerable degree of relationship to each other, but do not show complete cross reactions.

Neutralization Tests With Patients' Serums.—In the light of the demonstration of sharp strain differentiation with rabbit antiserums by the serum neutralization test, it was of interest to determine whether this more sensitive test might show greater specificity in the antibody response of the human host than did the agglutinin inhibition or complement fixation tests. Selected serums from patients studied during the 1947 outbreak of influenza were, therefore, tested for neutralizing antibody content, using the yolk sac method[9] for this purpose. The results are presented in Table III.

TABLE III. RESULTS OF NEUTRALIZATION TESTS WITH INFLUENZA VIRUSES AND SERUMS OF PATIENTS USING THE YOLK SAC ROUTE OF INFECTION

PATIENTS' SERUMS	VIRUS†				
	EB	GP	DW	FM_1	PR8
EB	<4/28*	—	<4/13	<4/87	7/137
GP	<4/32	<4/7	<4/8	<4/38	<4/16
DW	<4/<4	<4/<4	<4/<4	8/25	4/6
HB	<4/330	—	<4/131	<4/1024	<4/516
AA	<4/29	<4/<4	<4/64	4/128	201/720
DN	<4/4	<4/9	<4/4	<4/92	32/645
PW	<4/<4	—	<4/<4	<4/<4	<4/<4

—, Not done.
*Reciprocals of the serum dilutions giving 50 per cent protection (acute/convalescent).
†Inoculum per egg in most tests was about 50 LD_{50} (range 32-63) in five days.

One striking feature of the results obtained by this method in these patients' serums was the failure of the acute phase serums to neutralize any of the viruses to any significant extent. This is in contrast to the results obtained with the hemagglutination inhibition and complement fixation tests, but corresponds with the results of the neutralization tests in the normal rabbit serums when the allantoic method was used. There were two notable exceptions in that the acute phase serums of patients A. A. and D. N. had neutralizing titers of 1:201 and

1:32 respectively for the PR8 strain; low titers (1:4 to 1:8) with this strain were found also in the acute phase serums of two additional patients, and similar low titers were obtained in two patients with the FM_1 strain.

Among the epidemic cases, the neutralization of the viruses by the convalescent serums in this small group of adult patients failed to show clean-cut or consistent strain differences. In general, however, the highest titers and the greatest rises in the neutralizing titers occurred with the PR8 and the FM_1 strains, and this was true irrespective of whether the titers with the epidermic strains were high or low. The serums of D. W. failed to neutralize any of the local epidemic strains, including the one isolated from this patient's own throat washings, but they did show a slight and probably significant rise in titer with the FM_1 strain. One patient, A. A., failed to show any neutralizing antibodies for the GP strain, but showed a good antibody response to all of the other strains; in another of the epidemic cases, that of patient D. N., only minimal rises were demonstrated with the EB and DW strains, and only a slightly better response was obtained for the GP virus.

Results in a Sporadic Case.—The throat washings obtained from patient P. W. on Dec. 6, 1947, yielded a strain of influenza virus on several separate attempts. The virus was obtained on one occasion by amniotic inoculation and was first demonstrated in the third amniotic passage. It was also demonstrated on another occasion in the first amniotic passage of the lungs of mice that had been inoculated intranasally with the washings. Both of these strains were subsequently maintained by serial allantoic passage and they gave similar reactions in all tests. The strain obtained directly in eggs was used in the tests listed in Table II. As shown in Table II, this virus was neutralized only by its homologous rabbit antiserum, but not by the antiserums prepared with the Lee strain of influenza B, the 1947 epidemic strains, the 1943-1944 strains, nor with any of the standard influenza A strains. The acute and convalescent serum of P. W. failed to show any antibody response to any of the viruses used by the hemagglutination inhibition or complement fixation tests (Table I) or by the in ovo neutralization tests done by the yolk sac method (Table III).

DISCUSSION

The data presented indicate that the outbreak of influenza which occurred in Boston in March and April, 1947, was caused by strains of influenza virus which, though they resembled the PR8 strain of influenza A, were antigenically distinct from that strain and also from strains isolated in Boston during 1943-1944. Similar observations have now been recorded with respect to outbreaks of influenza which occurred during the same season in other parts of this country[1-3, 5] and in England.[4]

In addition, the strains isolated during this outbreak showed some antigenic differences among themselves, as indicated by cross neutralization tests in embryonated eggs with antiserums prepared in rabbits. Some differences also were noted in the antibody titers and rises in titer against these strains in the serums of patients, both in the in vitro tests used and, to some extent, in the in ovo neutralization tests done by the yolk sac method. Similar antigenic differences

also have been demonstrated among the strains isolated during the influenza epidemic in Boston in 1943-1944. The latter strains showed somewhat greater resemblance to the PR8 strain. Distinct antigenic differences among influenza B strains isolated during a single outbreak also have been demonstrated.[7]

Of particular interest were the occasional patients like D. W. who was ill during the height of the outbreak and P. W. who was ill in December when no other cases of influenza virus infection were established in this area. In these patients' serums little or no antibody response was detected, either with the standard strains of influenza virus or even with the strains isolated from their own throat washings. Similar observations previously have been made in several patients ill with clinical influenza shortly after the influenza A epidemic of 1943-1944.[6] Strains MA and CP were obtained from such cases during the latter outbreak.

In view of the distinct antigenic differentiation between the epidemic strains isolated in the spring of 1947 and the PR8 strain, it is also of interest that during this outbreak better antibody responses were elicited in the patients against the PR8 strain than against the strains isolated during this epidemic. This was true even in the patients from whom the viruses were isolated. Similar results were obtained during the same season in England.[4] In the present study this lack of specificity in the serologic response of adult patients was demonstrated, both by the in vitro tests (hemagglutination inhibition and complement fixation) and by the in ovo neutralization tests done by the yolk sac method. In the latter tests this was true in spite of the fact that all of the acute phase serums gave completely negative results with the three local epidemic strains. In the rabbit antiserums, on the other hand, the neutralization test permitted much more definite antigenic differentiation among the strains and proved much more satisfactory than in vitro tests for that purpose.

SUMMARY AND CONCLUSIONS

Strains of virus isolated from patients during the epidemic of influenza which occurred in Boston in March-April, 1947, were shown to be antigenically distinct from several classic strains of influenza A and from strains of influenza virus isolated in Boston during and shortly after the 1943-1944 epidemic. The strains from both of the epidemics, however, showed some antigenic relationship to the PR8 strain of influenza A, but not to the Lee strain of influenza B.

Evidence was presented which suggested that at least two antigenically distinct strains of influenza virus were active during the 1947 epidemic; one of them closely resembled the FM_1 strain isolated elsewhere during the same season. Antigenic differences were also demonstrated among strains of influenza A isolated in Boston during and after the outbreak of 1943-1944.

During the 1947 outbreak, higher titers and greater rises in titer of influenza antibodies were elicited in the serums of patients with the PR8 strain than with the epidemic strains.

A strain of virus isolated from a sporadic case of clinical influenza in December, 1947, showed no antigenic relationship to any of the influenza viruses included in this study. Antibodies against this strain could not be demonstrated in the serum of the patient from whom it was isolated.

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STUDIES ON CARDIOLIPIN ANTIGEN

IV. Variations in Sensitivity of Different Lots of Purified Lecithin

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IT WAS observed early that different lots of cardiolipin antigen prepared according to the same formula gave inconstant results in the standard Kahn test. The question arose whether the variable factor was present in the cardiolipin, the purified lecithin, or in both. This article briefly summarizes our serologic results based on the use of different lots of cardiolipin and lecithin.

Table I illustrates differences in sensitivity of two different lots of cardiolipin antigen in the standard Kahn test. The antigens were prepared according to a previously employed formula, namely, 0.75 per cent purified lecithin, 0.03 per cent cardiolipin, and 0.1 per cent cholesterol.[1] To this antigen was added 0.2 ml. of a 10 per cent solution of gum mastic (N. F.) in absolute alcohol. The titer with 0.9 per cent NaCl solution was the same with both antigens, namely, 1 plus 1.1. Standard Kahn tests were performed mainly with weakly positive syphilitic serums. Two readings of results were made, the first immediately after the addition of diluent (salt solution), and the second fifteen minutes later. A summation of plus signs of the readings of the three-tube tests with each antigen shows that cardiolipin antigen Lot P gave, on first and second readings, a total of 121 and 105 plus signs respectively, while antigen Lot L gave on first and second readings a total of 179 and 161 plus signs respectively. Thus, antigen Lot L was obviously more sensitive than antigen Lot P in the simultaneous examination of twenty-five serums.

This finding raised the question whether these differences in sensitivity were due to differences in cardiolipin or in purified lecithin. Table II illustrates standard Kahn results with antigens prepared with three different lots of cardiolipin and one lot of purified lecithin (Lot IA-46). Ten weakly positive serums were employed. The antigen containing cardiolipin Lot 42 gave, on first and second readings, a total of 45 and 42 pluses respectively. The antigen with cardiolipin Lot Run 3 gave, on first and second readings, a total of 43 and 42 pluses respectively. The antigen with cardiolipin Lot 35-37 gave, on first and second readings, 44 and 41 pluses respectively. It is thus evident that the three lots of antigen prepared with different lots of cardiolipin gave, with a single lot of purified lecithin, closely parallel Kahn results with weakly positive serums.

When turning to antigens containing different lots of purified lecithin with one lot of cardiolipin, the Kahn results were found to be of a different nature.

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TABLE I. ILLUSTRATING DIFFERENCES IN SENSITIVITY OF TWO DIFFERENT LOTS OF CARDIOLIPIN ANTIGEN PREPARED ACCORDING TO THE SAME FORMULA

	STANDARD KAHN REACTIONS WITH CARDIOLIPIN ANTIGEN EMPLOYING:			
	ANTIGEN LOT P		ANTIGEN LOT L	
	FIRST READING*	SECOND READING	FIRST READING	SECOND READING
	TUBES	TUBES	TUBES	TUBES
SERUM	1 2 3	1 2 3	1 2 3	1 2 3
1	2 4 4	1 4 4	4 4 4	4 4 4
2	- 4 4	- 4 4	4 4 4	4 4 4
3	- 4 4	- 4 4	3 4 4	3 4 4
4	- 4 4	- 4 4	3 4 4	3 4 4
5	- 4 4	- 2 3	3 4 4	1 4 4
6	- 4 4	- 2 2	1 4 4	- 4 4
7	- 3 4	- 3 4	3 4 4	3 4 4
8	- 3 4	- - 4	2 4 4	- 4 4
9	- 3 3	- 2 3	1 3 4	- 2 4
10	- 3 3	- 3 3	- 4 4	- 4 4
11	- 2 4	- 2 4	- 4 4	- 4 4
12	- 2 3	- 3 3	- 2 4	- - 4
13	- 1 4	- - 4	2 4 4	1 4 4
14	- 1 4	- 1 4	- 4 4	- 4 4
15	- ± 4	- ± 4	- 4 4	- 3 4
16	- - 3	- - 3	- 3 4	- 3 4
17	- 1 2	- - 1	- 2 3	- 2 3
18	- - 1	- - ±	- 2 3	- ± 2
19	- - 1	- - ±	- ± 1	- ± 1
20	- - -	- - -	- ± 3	- - 2
21	- - -	- - -	- - 3	- - ±
22-23	4 4 4	4 4 4	4 4 4	4 4 4
24-25	- - -	- - -	- - -	- - -
Total plus signs	121	105	179	161

*The first reading was made after the addition of diluent; the second reading was made fifteen minutes later.

TABLE II. ILLUSTRATING SIMILARITY IN SENSITIVITY OF CARDIOLIPIN ANTIGEN EMPLOYING LECITHIN LOT 1A-46 WITH THREE DIFFERENT LOTS OF CARDIOLIPIN

	STANDARD KAHN REACTIONS WITH CARDIOLIPIN ANTIGEN EMPLOYING:					
	CARDIOLIPIN 42		CARDIOLIPIN RUN 3		CARDIOLIPIN 35-37	
	FIRST READING*	SECOND READING	FIRST READING	SECOND READING	FIRST READING	SECOND READING
	TUBES	TUBES	TUBES	TUBES	TUBES	TUBES
SERUM	1 2 3	1 2 3	1 2 3	1 2 3	1 2 3	1 2 3
1	- 1 3	- 1 2	- 1 3	- 1 2	- 1 3	- 1 3
2	- 2 3	- 2 2	- 1 3	- 2 3	- 2 3	- 2 3
3	- - 2	- - 2	- 1 1	- 1 1	- - 2	- - 2
4	- - 1	- - 1	- - ±	- - ±	- - ±	- - ±
5	- 3 4	- 2 4	- 3 4	- 3 3	- 2 4	- 2 4
6	- 1 3	- 2 2	- 2 3	- 2 3	- 2 2	- 1 1
7	- - 1	- - 1	- - ±	- - ±	- - 1	- - 1
8	1 4 4	1 4 4	1 4 4	1 4 4	2 4 4	1 4 4
9	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4
10	- - -	- - -	- - -	- - -	- - -	- - -
Total plus signs	45	42	43	42	44	41

*The first reading was made after addition of the diluent; the second reading was made fifteen minutes later.

as is evident from Table III. An antigen containing purified lecithin Lot 1A-46 gave, on first and second readings, 41 and 33 pluses respectively. An antigen containing lecithin Lot Run 4 gave, on first and second readings, 69 and 65 pluses respectively.

TABLE III. ILLUSTRATING DIFFERENCES IN SENSITIVITY OF CARDIOLIPIN (P) ANTIGEN WITH TWO DIFFERENT LOTS OF PURIFIED LECITHIN

	STANDARD KAHN REACTIONS WITH CARDIOLIPIN ANTIGEN EMPLOYING:			
	LECITHIN 1A-46		LECITHIN RUN 4	
	FIRST READING*	SECOND READING	FIRST READING	SECOND READING
SERUM	TUBES 1 2 3	TUBES 1 2 3	TUBES 1 2 3	TUBES 1 2 3
1	– 1 3	– – 2	– 3 4	– 3 4
2	– 1 3	– – –	2 4	– 2 4
3	– – 3	– – 2	– 3 3	– 2 2
4	– ± 2	– ± 2	3 4 4	3 4 4
5	– 3 4	– 2 4	– 4 4	– 4 4
6	– 1 3	– 2 2	– 3 4	– 2 3
7	– 4 4	– 4 4	3 4 4	3 4 4
8	– – –	– – –	– 1 1	– 1 1
9	2 3 4	2 3 4	3 4 4	3 4 4
10	– – –	– – –	– – –	– – –
Total plus signs	41	33	69	65

*The first reading was made after the addition of diluent; the second reading was made fifteen minutes later.

TABLE IV. ILLUSTRATING DIFFERENCES IN SENSITIVITY OF CARDIOLIPIN (L) ANTIGEN WITH THREE DIFFERENT LOTS OF PURIFIED LECITHIN

	STANDARD KAHN REACTIONS WITH CARDIOLIPIN ANTIGEN EMPLOYING:					
	LECITHIN 1A-46		LECITHIN RUN 3		LECITHIN RUN 4	
	FIRST READING*	SECOND READING	FIRST READING	SECOND READING	FIRST READING	SECOND READING
SERUM	TUBES 1 2 3	TUBES 1 2 3	TUBES 1 2 3	TUBES 1 2 3	TUBES 1 2 3	TUBES 1 2 3
1	– 2 3	– – 3	– 4 4	– 4 4	– 3 4	– – 4
2	– 3 4	– 3 3	– 4 4	– 4 4	– 4 4	– 4 4
3	– 1 4	– 2 4	– 4 4	– 4 4	– 4 4	– 3 4
4	– 2 3	– 2 3	– 4 4	– 3 4	– 3 4	– 3 4
5	– 1 3	– – 3	– 4 4	– – 4	– 2 4	– 1 3
6	– – –	– – –	0 0 0	0 0 0	– – 3	– – 3
7	– – –	– – –	– 2 4	– 1 4	– – 1	– – 1
8	– – –	– – –	– 1 4	– – 3	– – –	– – –
9	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4
10	– – –	– – –	– – –	– – –	– – –	– – –
Total plus signs	38	35	63	55	52	46

*The first reading was made after addition of the diluent; the second reading was made fifteen minutes later.

TABLE V. ILLUSTRATING DIFFERENCES IN SENSITIVITY OF TWO DIFFERENT LOTS OF CARDIOLIPIN ANTIGEN PREPARED ACCORDING TO THE SAME FORMULA—WITHOUT THE ADDITION OF MASTIC

	STANDARD KAHN REACTIONS WITH CARDIOLIPIN ANTIGEN EMPLOYING:	
	LECITHIN LOT 17	LECITHIN LOT 11
SERUM	TUBES 1 2 3	TUBES 1 2 3
1	± 3 4*	1 4 4
2	± 4 4	– 3 4
3	– 3 4	– 3 4
4	– – 1	– 3 4
5	– – ±	– 3 4
6	– 4 4	– 3 4
7	– 2 4	– 2 4
8	– ± ±	– ± 3
9	– – –	– – 3
10	– – –	– – –
Total plus signs	37	56

*One reading of the tests was made, namely immediately after the addition of diluent.

It seemed worth while to serologically examine different antigens containing different lots of lecithin with still another lot of cardiolipin, Lot L, instead of Lot P employed in the previous experiment. Table IV summarizes the results of this comparison. The antigen with lecithin Lot 1A-46 gave in the Kahn test 38 pluses on the first reading and 35 pluses on the second reading. The antigen with lecithin Lot Run 3 gave 63 pluses on the first reading and 55 pluses on the second reading. The antigen with lecithin Lot Run 4 gave, on first and second readings, 52 and 46 pluses respectively.

A similar experiment in which two different lots of lecithin were employed with two different lots of cardiolipin in accordance with our latest formula,[2] in which 1.0 per cent lecithin is employed with 0.1 per cent cardiolipin and 0.025 per cent cholesterol, is illustrated in Table V. No mastic was employed in this formula. It is evident from Table V that cardiolipin antigen containing Lecithin Lot 11 gave, with ten weakly positive serums, a total of 56 plus signs, while cardiolipin antigen containing lecithin Lot 17 gave with the same serums a total of 37 plus signs.

It is thus clear that different lots of purified lecithin in cardiolipin antigen may cause the antigen to vary in sensitivity to the extent of 40 per cent or more. These differences can be noted best when weakly positive reacting serums are employed.

Preliminary standardization methods developed in this laboratory for cardiolipin antigen indicate that many purified lecithins will become usable by variations in the ratio of lecithin to cardiolipin within certain limits. Thus with the employment of a 10:1 ratio of lecithin: cardiolipin as a working base, a reduction in lecithin, such as 9:1, tends to decrease sensitivity, while an inincrease in lecithin, such as 11:1, tends to increase sensitivity.[3]

SUMMARY

It was observed that different cardiolipin antigens, prepared according to the same formula but consisting of different lots of cardiolipin, were of uniform sensitivity in the standard Kahn test. When the cardiolipin antigens were prepared with different lots of purified lecithin, considerable variations in sensitivity were noted. This finding indicates that cardiolipin antigens prepared with different lots of purified lecithin require serologic standardization before their employment in tests for syphilis.

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STUDIES ON CARDIOLIPIN ANTIGEN

V. Standardization of Antigen for the Kahn Test

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THIS article deals with various aspects of the standardization of cardiolipin antigen for the standard Kahn test. Preliminary studies with this antigen indicated that it behaves essentially like Kahn antigen in its titration with salt solution. These studies further indicated that in the final standardization of cardiolipin antigen for use with serum this antigen also behaves essentially like Kahn antigen. Hence, many technical steps developed in connection with the titration and standardization of Kahn antigen were found to be applicable to the titration and standardization of cardiolipin antigen. The major aim of standardization of cardiolipin antigen is of course the same as that of the standardization of Kahn antigen, namely, to assure uniformity in serologic results with syphilitic and nonsyphilitic serums.

The Need of Standardization of Cardiolipin Antigen.—Tissue extract antigen, such as Kahn antigen, requires three steps in its standardization. (1) The titration of the antigen with salt solution to determine the titer at which the antigen is to be mixed with salt solution in the preparation of the antigen suspension for use with serum. (2) Comparative tests with syphilitic and nonsyphilitic serums, employing standard antigen as a control, to determine how closely a new antigen behaves like standard antigen in sensitivity and specificity. (3) Adjustment or correction of the new antigen if in its behavior with serums it is either oversensitive or undersensitive as compared with standard antigen. These three steps are necessary also in the standardization of cardiolipin antigen.

The question might arise why an antigen consisting of purified chemical reagents should need to be standardized. The need for standardization of cardiolipin antigen was pointed out in the preceding article,[1] namely, different lots of purified lecithin do not give rise to identical serologic results.

A Workable Cardiolipin Antigen Formula for the Standard Kahn Test.—In attempting to develop a workable cardiolipin antigen formula for the standard Kahn test, various ratios of lecithin-cardiolipin were tried. A 25:1 ratio of these reagents first was reported with the use of a very small amount of mastic.[2] With the abandonment of this colloid, that ratio was found to be unsuitable for the standard Kahn test. A 20:1 ratio of lecithin-cardiolipin was then tried. Tabular results of cardiolipin antigens prepared with this ratio in their behavior with salt solution will be presented later. At present it is merely desired to state that a 10:1 ratio of lecithin-cardiolipin was found to be suitable as a working base, provided the concentration of these reagents is taken into consideration.[3, 4]

From the Serology Laboratory, University Hospital, University of Michigan.
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Cardiolipin and purified lecithin employed in this study were kindly supplied by the Lederle Laboratories, Pearl River, N. Y., and by Dr. Mary Pangborn, New York State Department of Health, Albany, N. Y.
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The proper concentration of lipids plays an important role in the behavior of Kahn antigen with salt solution and with serums, and it is understandable that it would also play an important role in the behavior of cardiolipin antigen. Indeed, several years' trial of adjusting cardiolipin antigen to the standard Kahn test led to failure until proper concentration of reagents began to be employed. The relationship between lecithin-cardiolipin ratios and the concentration of these reagents will be discussed below. First it is desired to present the basic cardiolipin antigen formula employed.

The use of the following percentages of cardiolipin, purified lecithin, and cholesterol results in a cardiolipin antigen which behaves broadly like Kahn antigen, both in its titration with salt solution and in its reactions with serum:

Purified lecithin	1.0 per cent
Cardiolipin	0.1 per cent
Cholesterol	0.025 per cent

An outstanding feature of this formula is the relatively high concentrations of cardiolipin and lecithin and the very low concentration of cholesterol. It is believed that the 25 mg. per cent of cholesterol in cardiolipin antigen matched against the 600 mg. per cent of cholesterol in Kahn antigen might help to bring out selective reactivities of the two antigens in certain situations in syphilis.

Determination of Cardiolipin Antigen Titer.—Those who are familiar with the Kahn technique[5] will recall that when 1 ml. of the antigen is mixed with an appropriate amount of salt solution, an antigen suspension is produced containing lipid aggregates. An important characteristic of these aggregates is that they are dispersible in salt solution and in serum; then, in syphilitic serum new floccules appear, while in nonsyphilitic serum no floccules appear. Essentially, cardiolipin antigen behaves the same way with salt solution. When appropriately mixed with salt solution, an antigen suspension is produced in which the lipid aggregates are dispersible in salt solution and in serum. Then, as in the case of the Kahn antigen suspension, floccules appear in syphilitic serum and no floccules appear in nonsyphilitic serum.

The titer of Kahn antigen, by definition, is the smallest amount of salt solution added to 1 ml. of antigen which will produce a lipid suspension containing dispersible aggregates. The same definition applies to cardiolipin antigen. However, after the titer has been obtained, it is not necessarily true that either antigen at the titer will give correct results with serum. The sensitivity of the titered antigens with serums must be determined by trial.

Briefly, the basic requirements of an antigen suspension, at its titer, for use in tests with serum are as follows.

(1) The antigen suspension must contain a minimal amount of salt solution; an increase in the salt solution beyond the titer tends to reduce sensitivity and a decrease in the salt solution below the titer tends to increase sensitivity and nonspecificity.

(2) The lipid aggregates of the antigen suspension must disperse in salt solution and in serum.

(3) When these aggregates are thus dispersed in serum, the negative reactions appear opalescent—not suggestive of cloudiness on the one hand or of water clarity on the other. The positive reactions will then show floccules of sufficient bulk as to be readily differentiable.

(4) If an antigen at its titer does not give results comparable to standardized Kahn antigen, special standardization methods are applied with a view toward bringing the antigen to standard sensitivity.

Table I illustrates the titration pictures of cardiolipin and Kahn antigens. The similarity in the titration picture of two antigens is evident. Also evident is the fact that cardiolipin antigen exhibits a narrow titration range with salt solution, while Kahn antigen exhibits a relatively wide titration range. As is illustrated in Table I, the Kahn antigen has a titer of 1 plus 1.3, while the cardiolipin antigen has a titer of 1 plus 0.9. When 1 ml. of cardiolipin antigen is mixed with 0.8 ml. salt solution instead of 0.9 ml., the resulting antigen suspension gives cloudy mixtures with serum and it is impossible to differentiate syphilitic from nonsyphilitic serums. If 1 ml. of the antigen is mixed with 1 ml. of salt solution, the resulting antigen suspension gives altogether too clear mixtures with serum and the suspension is of markedly reduced sensitivity. Briefly, cardiolipin antigen must be mixed with salt solution precisely at the titer, namely 1 plus 0.9, to obtain a usable antigen suspension in which syphilitic serums will show precipitates and nonsyphilitic serums will show the opalescence and clarity characteristic of negative reactions.

TABLE I. SIMILARITY OF TITRATION PICTURES OF CARDIOLIPIN AND KAHN ANTIGENS

CARDIOLIPIN ANTIGEN SUSPENSIONS: AMOUNTS OF ANTIGEN AND 0.9 PER CENT SALT SOLUTION (ML.)	DISPERSIBILITY OF ANTIGEN SUSPENSION AGGREGATES; RESULTS IN 3-TUBE TEST, USING SALT SOLUTION INSTEAD OF SERUM	KAHN ANTIGEN SUSPENSIONS: AMOUNTS OF ANTIGEN AND 0.9 PER CENT SALT SOLUTION (ML.)
1 + 0.8	Cloudy; nondispersible aggregates	1 + 1.1
1 + 0.9*	Opalescent (titer); dispersible aggregates	1 + 1.3*
1 + 1.0	Too clear; dispersible aggregates	1 + 1.5
1 + 1.1	Much too clear; dispersible aggregates	1 + 1.7
1 + 1.2	Water clear; dispersible aggregates	1 + 1.9

*Antigen titers employed in the Kahn test.
Cardiolipin antigen Lot C11, L16-AA; Kahn antigen Lot 107A.

Kahn antigen, on the other hand, is likely to give results closely similar to those given at a titer of 1 plus 1.3 when the antigen suspensions are prepared by adding 1.2 ml. salt solution to 1 ml. of antigen or 1.4 ml. of salt solution to 1 ml. of antigen. Briefly, 0.1 ml. of salt solution above or below the titration end point apparently does not markedly affect the physical properties of Kahn antigen suspensions.

This difference between Kahn and cardiolipin antigen is based on the probability that Kahn antigen contains nonantigenic colloids which are protective in nature, while cardiolipin antigen, because of its high purity, lacks these protective colloids. Fig. 1 graphically illustrates the narrow titration range of cardiolipin antigen as compared with the relatively wide titration range of Kahn antigen.

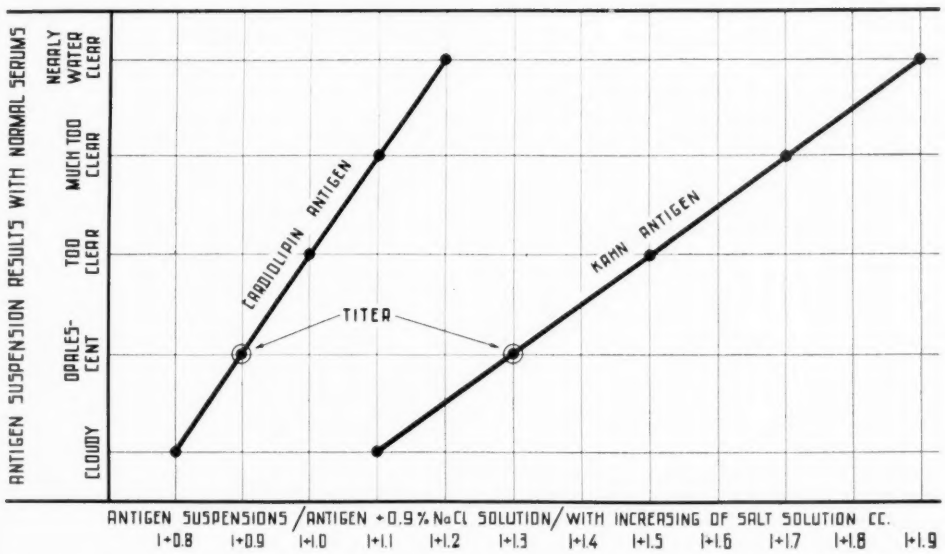
ANTIGEN SUSPENSION RESULTS WITH NORMAL SERUMS
CLOUDY
OPALES-CENT
TOO CLEAR
MUCH TOO CLEAR
NEARLY WATER CLEAR
CARDIOLIPIN ANTIGEN
KAHN ANTIGEN
TITER
ANTIGEN SUSPENSIONS / ANTIGEN + 0.9% NaCl SOLUTION / WITH INCREASING OF SALT SOLUTION CC.
1+0.8 1+0.9 1+1.0 1+1.1 1+1.2 1+1.3 1+1.4 1+1.5 1+1.6 1+1.7 1+1.8 1+1.9

Fig. 1.—Graph illustrating antigen suspension of correct titer in relation to suspensions that give either too clear or too cloudy results.

Table II shows that a titer of cardiolipin antigen prepared in a 10:1 ratio of lecithin-cardiolipin is obtainable only under conditions of the appropriate concentration of these reagents. When employing the same ratios of lecithin-cardiolipin, namely 10:1, but in different concentrations of these reagents, workable titers are not obtained. For example, 0.5 per cent of lecithin and 0.05 per cent of cardiolipin do not lead to a workable titer. The same holds true if 2.0 per cent lecithin and 0.2 per cent cardiolipin are employed. Evidently the 10:1 ratio of lecithin to cardiolipin in combination with the appropriate concentration of these reagents is of importance in obtaining a workable cardiolipin antigen titer for the Kahn test.

TABLE II. CONCENTRATIONS OF LECITHIN AND CARDIOLIPIN (10:1 RATIO) PRODUCING AN ANTIGEN SUSPENSION USABLE IN THE STANDARD KAHN TEST

ANTIGEN PLUS SALT SOLUTION (ML.)	CARDIOLIPIN ANTIGEN FORMULA		
	A 0.5% LECITHIN 0.05% CARDIOLIPIN 0.025% CHOLESTEROL	B 1.0% LECITHIN 0.1% CARDIOLIPIN 0.025% CHOLESTEROL	C 2.0% LECITHIN 0.2% CARDIOLIPIN 0.025% CHOLESTEROL
	TOTAL LIPID CONCENTRATION		
	0.575%	1.125%	2.225%
1 + 0.8	Too clear; some nondispersible aggregates	Cloudy; nondispersible aggregates	Turbid; nondispersible aggregates
1 + 0.9	Nearly water-clear; dispersible aggregates	Opalescent*; dispersible aggregates	Turbid; nondispersible aggregates
1 + 1.0	Water clear; dispersible aggregates	Too clear; dispersible aggregates	Cloudy; nondispersible aggregates
1 + 1.1	Water clear; dispersible aggregates	Much too clear; dispersible aggregates	Cloudy; some nondispersible aggregates
1 + 1.2	Water clear; dispersible aggregates	Nearly water clear; dispersible aggregates	Opalescent; some nondispersible aggregates

*A usable antigen suspension was obtained with Formula B when employing 1 ml. antigen plus 0.9 ml. salt solution.

Table III illustrates that a 20:1 ratio of lecithin-cardiolipin does not produce a workable titer at any of the three concentrations of the reagents employed, namely 0.5 per cent and 0.025 per cent, 1.0 per cent and 0.05 per cent, and 2.0 per cent and 0.1 per cent of lecithin to cardiolipin respectively.

TABLE III. CONCENTRATIONS OF LECITHIN AND CARDIOLIPIN (20:1 RATIO) NOT PRODUCING AN ANTIGEN SUSPENSION USABLE IN THE STANDARD KAHN TEST

ANTIGEN PLUS SALT SOLUTION (ML.)	CARDIOLIPIN ANTIGEN FORMULA		
	D 0.5% LECITHIN 0.025% CARDIOLIPIN 0.025% CHOLESTEROL	E 1.0% LECITHIN 0.05% CARDIOLIPIN 0.025% CHOLESTEROL	F 2.0% LECITHIN 0.1% CARDIOLIPIN 0.025% CHOLESTEROL
	TOTAL LIPID CONCENTRATION		
	0.55%	1.075%	2.125%
1 + 0.8	To clear; some nondispersible aggregates	Opalescent; nondispersible aggregates	Turbid; nondispersible aggregates
1 + 0.9	Nearly water clear; some nondispersible aggregates	Too clear; nondispersible aggregates	Turbid; nondispersible aggregates
1 + 1.0	Water clear; some nondispersible aggregates	Too clear; some nondispersible aggregates	Cloudy; nondispersible aggregates
1 + 1.1	Water clear; some nondispersible aggregates	Nearly water clear; some nondispersible aggregates	Opalescent; nondispersible aggregates
1 + 1.2	Water clear; dispersible aggregates	Water clear; some nondispersible aggregates	Too clear; some nondispersible aggregates

Table IV shows the effect of increasing the cholesterol in a usable cardiolipin antigen formula on the titration results. It was found that 0.025 per cent of cholesterol did not interfere with the titration readings and was therefore adopted for use in the antigen formula. Certain lots of cardiolipin and lecithin

TABLE IV. EFFECT OF INCREASING CHOLESTEROL IN USABLE CARDIOLIPIN ANTIGEN FORMULA ON TITRATION PICTURE

ANTIGEN PLUS SALT SOLUTION (ML.)	AMOUNT OF CHOLESTEROL ADDED TO 1% LECITHIN AND 0.1% CARDIOLIPIN (10:1 RATIO)				
	0%	0.025%	0.05%	0.1%	0.2%
1 + 0.8	Cloudy; nondispersible aggregates	Cloudy; nondispersible aggregates	Cloudy; nondispersible aggregates	Cloudy; nondispersible aggregates	Cloudy; nondispersible aggregates
1 + 0.9	Opalescent; slightly too clear; dispersible aggregates	Opalescent*; dispersible aggregates	Opalescent; trace nondispersible aggregates	Opalescent; some non-aggregates dispersible	Opalescent; some nondispersible aggregates
1 + 1.0	Too clear; dispersible aggregates	Too clear; dispersible aggregates	Too clear; dispersible aggregates	Too clear; aggregates dispersible	Too clear; some nondispersible aggregates
1 + 1.1	Water clear; dispersible aggregates	Water clear; dispersible aggregates	Water clear; dispersible aggregates	Nearly water clear; dispersible aggregates	Nearly water clear; trace nondispersible aggregates
1 + 1.2	Water clear; dispersible aggregates	Water clear; dispersible aggregates	Water clear; dispersible aggregates	Water clear; dispersible aggregates	Water clear; dispersible aggregates

*A usable antigen suspension was obtained when employing 1 ml. antigen plus 0.9 ml. salt solution.

may be found usable with 0.05 per cent cholesterol, but none thus far have given satisfactory titration results with 1.0 per cent cholesterol.

The Titer in Relation to the Sensitivity of Cardiolipin Antigen.—It was already indicated that an antigen at its titer may not necessarily give correct sensitivity results. Table V shows that cardiolipin antigens, containing lecithins not approved by Dr. Pangborn,* may give titration results identical to those of antigens containing approved lots of lecithin. It is evident from Table V that lecithin Lots 12A and 13 gave satisfactory titration results similar to results of approved lots of lecithin.

TABLE V. UNIFORMITY OF TITRATION RESULTS OF SEVEN LOTS OF CARDIOLIPIN ANTIGEN, EMPLOYING SEVEN DIFFERENT LOTS OF CARDIOLIPIN AND FIVE DIFFERENT LOTS OF LECITHIN; FORMULA: 1 PER CENT LECITHIN, 0.1 PER CENT CARDIOLIPIN, AND 0.025 PER CENT CHOLESTEROL

CARDIOLIPIN ANTIGEN LOT	DEGREE OF DISPERSIBILITY OF LIPID AGGREGATES OF ANTIGEN SUSPENSION				
	AGGREGATES NONDISPERSIBLE	AGGREGATES DISPERSIBLE; OPALESCENT (TITER)	AGGREGATES DISPERSIBLE; TOO CLEAR	AGGREGATES DISPERSIBLE; MUCH TOO CLEAR	AGGREGATES DISPERSIBLE; WATER CLEAR
	Antigen plus Salt Solution (ml.)				
C9:L11	1 + 0.8	1 + 0.9	1 + 1.0	1 + 1.1	1 + 1.2
C3:LIV	1 + 0.8	1 + 0.9	1 + 1.0	1 + 1.1	1 + 1.2
C35-37:L4	1 + 0.8	1 + 0.9	1 + 1.0	1 + 1.1	1 + 1.2
C13:L12A*	1 + 0.8	1 + 0.9	1 + 1.0	1 + 1.1	1 + 1.2
C6R:L13*	1 + 0.8	1 + 0.9	1 + 1.0	1 + 1.1	1 + 1.2
C8:L13*	1 + 0.8	1 + 0.9	1 + 1.0	1 + 1.1	1 + 1.2
C11:L13*	1 + 0.8	1 + 0.9	1 + 1.0	1 + 1.1	1 + 1.2

*Lecithin not approved by Dr. Pangborn.

The differences in sensitivity of cardiolipin antigens, prepared with different lots of lecithin, having the same titer of 1 plus 0.9 (Table V) are illustrated in Table VI. It is evident from Table VI that cardiolipin antigens prepared with lecithin Lots 12A and 13 are below the sensitivity of the other lecithin lots.

TABLE VI. COMPARATIVE SENSITIVITY OF FIVE LOTS OF CARDIOLIPIN ANTIGEN, EMPLOYING FIVE DIFFERENT LOTS OF CARDIOLIPIN AND FOUR DIFFERENT LOTS OF LECITHIN; FORMULA: 1.0 PER CENT LECITHIN, 0.1 PER CENT CARDIOLIPIN, AND 0.025 PER CENT CHOLESTEROL

SERUM	LECITHIN LOT 11 / CARDIOLIPIN LOT 9 / ANTIGEN TITER 1 + 0.9 (CONTROL)	LECITHIN LOT IV / CARDIOLIPIN LOT 3 / ANTIGEN TITER 1 + 0.9	LECITHIN LOT 12A* / CARDIOLIPIN LOT 13* / ANTIGEN TITER 1 + 0.9	LECITHIN LOT 13* / CARDIOLIPIN LOT 8 / ANTIGEN TITER 1 + 0.9	LECITHIN LOT 13* / CARDIOLIPIN LOT 11 / ANTIGEN TITER 1 + 0.9	KAHN STANDARD ANTIGEN LOT 107A / ANTIGEN TITER 1 + 1.3 (CONTROL)
1	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4
2	4 4 4	4 4 4	3 4 4	3 3 4	± 3 4	4 4 4
3	3 4 4	3 4 4	3 4 4	2 2 3	– – ±	4 4 4
4	2 4 4	2 4 4	– 3 4	± 2 2	2 2 2	± 4 4
5	3 4 4	3 4 4	± 2 2	– ± ±	± ± ±	– 2 4
6	4 4 4	4 4 4	± ± 1	– – ±	– – –	3 4 4
7	– 4 4	± 4 4	± ± ±	– ± ±	– – –	– 4 4
8	– 2 4	– 2 4	– – 2	– – 1	– 1 2	– 3 4
9	– – ±	– – ±	– – –	– – –	– – –	– – 4
10	– – ±	– – –	– – –	– – –	– – –	– – 3

*Not approved by Dr. Pangborn.

*New York State Department of Health.

In attempting to bring the sensitivity of different lots of cardiolipin antigen to a standard level, it was observed that this can be achieved with certain lots of lecithin by merely altering the ratio of lecithin-cardiolipin. If instead of employing a 10:1 ratio of lecithin-cardiolipin, an 11:1 ratio is employed, increased sensitivity is generally obtained. If, on the other hand, a 9:1 ratio of lecithin-cardiolipin is employed, the sensitivity is generally decreased as compared with the 10:1 ratio. Table VII illustrates the sensitivity results obtained on increasing the amount of lecithin above the 10:1 ratio and on decreasing the amount of lecithin below this ratio.

TABLE VII. EFFECT OF VARYING LECITHIN-CARDIOLIPIN RATIOS ON SENSITIVITY; FORMULAS: 0.8, 0.9, 1.0, 1.1, AND 1.2 PER CENT LECITHIN (LOT 11), RESPECTIVELY, 0.1 PER CENT CARDIOLIPIN (LOT 9), AND 0.025 PER CENT CHOLESTEROL

	LECITHIN-CARDIOLIPIN RATIO				
	8:1	9:1	10:1 (CONTROL)	11:1	12:1
	ANTIGEN TITER				
SERUM	1 + 0.9	1 + 0.9	1 + 0.9	1 + 0.9	1 + 0.9
	Syphilitic Serums				
1	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4
2	3 4 4	3 4 4	3 4 4	4 4 4	4 4 4
3	1 1 1	1 2 3	3 4 4	3 4 4	4 4 4
4	± 4 4	± 4 4	3 4 4	3 4 4	4 4 4
5	2 3 3	2 3 4	2 4 4	2 4 4	3 4 4
6	± 4 4	± 4 4	1 4 4	1 4 4	3 4 4
7	− − 4	− 3 4	2 3 4	3 4 4	2 4 4
8	1 2 2	1 2 3	− 2 3	± 2 3	− 3 4
9	− − −	− − ±	− ± 1	− ± 3	− ± 3
10	− − ±	− − ±	− − ±	− − 3	− − 3
	Nonsyphilitic Serums				
11	− − −	− − −	− − −	± − −	± ± ±
12	− − −	− − −	− − −	± − −	± ± ±
13	− − −	− − −	− − −	− − −	− ± ±
14	− − −	− − −	− − −	− − −	± ± ±
15	− − −	− − −	− − −	− − −	± ± ±

Occasionally it may be found that the change in the lecithin-cardiolipin ratios from 10:1 to 9:1 or 11:1 may lead to sensitivity results beyond those of standard sensitivity. In such instances one should try ratios of 9.5:1 or 10.5:1 as the case may be. Table VIII illustrates the correction of a new lot of cardiolipin antigen, undersensitive in a 10:1 ratio, by employing a 10.7:1 ratio.

It is believed that modification of the lecithin-cardiolipin ratio will not, by itself, correct all undersensitive cardiolipin antigens. It is likely that a small change in the titer, such as a slight reduction in the amount of salt solution in the preparation of the antigen suspension, will increase sensitivity. An increase in the amount of cholesterol in the antigen should also increase sensitivity. Furthermore, adjustment of the concentrations of the lecithin and cardiolipin should play a role in sensitivity. These several steps should make possible the bringing of certain undersensitive cardiolipin antigens to standard sensitivity. In view of the limited availability of cardiolipin antigens with different lecithins, it has not yet been possible to investigate all methods which may increase the sensitivity of cardiolipin antigen. It should be added that cardiolipin antigen in the standard Kahn test, in the present state of our knowledge of this antigen, is employed in this laboratory only supplementary to Kahn antigen.

TABLE VIII. EFFECT OF VARYING LECITHIN-CARDIOLIPIN RATIOS ON SENSITIVITY AS A METHOD IN ANTIGEN STANDARDIZATION; FORMULAS: 1.0 AND 1.07 PER CENT LECITHIN (LOT 17), RESPECTIVELY, 0.1 PER CENT CARDIOLIPIN (LOT 11), AND 0.025 PER CENT CHOLESTEROL

	LECITHIN		
	LOT 17	LOT 17	LOT 11
	CARDIOLIPIN		
	LOT 11	LOT 11	LOT 9
	LECITHIN-CARDIOLIPIN RATIO*		
SERUM	10:1	10.7:1	10:1 (CONTROL)
	Syphilitic Serums		
1	±11	±12	±11
2	−±±	−±1	−12
3	±11	±23	±23
4	−±±	±33	±33
5	−34	±34	±24
6	−−4	−24	−24
7	±34	±34	±34
8	−−1	±44	−34
9	±34	±34	±34
10	−24	±34	±34
11	±33	134	144
12	444	444	444
	Nonsyphilitic Serums		
13-24	−−−	−−−	−−−

*The antigen titer in each case was 1 + 0.9.

SUMMARY

Standardization methods applicable to Kahn antigen for use in the standard Kahn test are broadly applicable also to cardiolipin antigen for use in this test. The titer of cardiolipin antigen with salt solution is obtained according to the same procedure as the Kahn antigen titer. The technique for establishing the sensitivity of cardiolipin antigen with syphilitic and nonsyphilitic serums is also similar to the technique for establishing the sensitivity of Kahn antigen. Techniques for the standardization of cardiolipin antigen to help provide uniformity in results are presented in this article.

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PROCAINE PENICILLIN; THERAPEUTIC EFFICIENCY AND A COMPARATIVE STUDY OF THE ABSORPTION OF SUSPENSIONS IN OIL AND IN OIL PLUS ALUMINUM MONOSTEARATE AND OF AN AQUEOUS SUSPENSION CONTAINING SODIUM CARBOXYMETHYLCELLULOSE

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ONE of the greatest disadvantages of penicillin is its rapid elimination from the body. In order to decrease the frequency of injections many attempts have been made to delay the absorption or excretion of penicillin and thus prolong the action of an injected dose. Until recently the most successful method of prolonging the concentrations of penicillin in the blood has been the incorporation of penicillin in peanut oil and beeswax.[1] With this preparation a single injection of 1 c.c. containing 300,000 units of penicillin was usually followed by assayable blood concentrations for twenty-four hours in 90 to 92 per cent of patients. The introduction of a fluid preparation obviated some of the difficulties inherent in the administration of this material.[2] Fluid penicillin in peanut oil and beeswax was found to be as effective as the original viscid preparation when 50 per cent of the total relative weight is made up of particles of 50 μ or more in length.[3] Discomfort to the patient in the form of local pain, tenderness, and nodule formation at the site of injection, however, still persisted.

For some time it has been known that a mixture of concentrated solutions of penicillin and procaine resulted in the formation of crystals which were identified as the procaine salt of penicillin. Whereas the commercially available salts (sodium, potassium, and calcium) are highly soluble in aqueous solutions and body fluids, the procaine salt is relatively insoluble. This property forms the basis of a new principle of penicillin administration. As a result of the low solubility of procaine penicillin, a repository injection of a suspension of this salt in oil or water results in delayed absorption and prolonged blood concentrations. This report presents the results of our studies on absorption following the intramuscular injection of procaine penicillin and the treatment of patients with various infections when procaine penicillin in oil was used.

MATERIALS

Crystalline procaine penicillin is usually prepared by the double decomposition of sodium penicillin G and procaine hydrochloride. The original commercial preparations were suspended in refined sesame or peanut oil so that 300,000 units were present in 1 c.c. as a free-flowing fluid material. Such a preparation need not be refrigerated since it will remain stable for at least one year at room temperature. Because the procaine penicillin on standing separates from the oil and because of the necessity for vigorous agitation in

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order to re-establish the suspension, detergents such as Tween 80* and Span 80† have been added to facilitate resuspension. These measures have been only partially successful. Other investigators[4] have added aluminum monostearate to the mixture to maintain the suspension. The addition of aluminum monostearate to procaine penicillin in oil results in a gel formation which maintains the suspension of the penicillin in the oil.

Peanut or sesame oil was originally employed as a vehicle for injecting procaine penicillin since it was impossible to prepare injectable water suspensions of this penicillin salt. Recently it has been demonstrated that the addition of dried sodium carboxymethylcellulose to dry crystalline procaine penicillin results in a stable suspension in diluents containing water.[5] Sodium carboxymethylcellulose in aqueous solution forms a viscous gel which maintains the procaine penicillin in discrete particulate suspension. This has eliminated the necessity for the use of oils, which have been shown to be antigenic and which may cause serious complications if they are injected accidentally into a blood vessel.[6]

All of the procaine penicillin preparations can be withdrawn from the vial and administered through a 19 or 20 gauge needle. For the preparations containing aluminum monostearate or sodium carboxymethylcellulose, dry syringes and needles are not needed. When procaine penicillin in oil is administered, moist needles and syringes may be used if the injection is made immediately after the syringe is filled. Although we have given multiple injections of procaine penicillin in oil from a single syringe without difficulty provided the withdrawal and injections were made within a very few minutes, these precautions are not necessary with the preparations containing aluminum monostearate or sodium carboxymethylcellulose.

STUDIES ON ABSORPTION

The concentrations of penicillin in the blood at various intervals were determined according to the method of Randall and associates[7] following the intramuscular injection of (1) procaine penicillin in oil,‡ (2) procaine penicillin in oil plus aluminum monostearate,§ and (3) procaine penicillin plus sodium carboxymethylcellulose in aqueous suspension.‖ The results are expressed both as percentage of patients having assayable concentrations (.03 units per cubic centimeter or more) and as the median concentrations at the various intervals tested.

As shown in Table I, all of the patients who received a single or initial injection of 300,000 units of procaine penicillin in oil (1 c.c.) had detectable levels at one, four, twelve, sixteen, and twenty hours. Only an occasional patient failed to have an assayable level at the twenty-fourth hour. About one-half of the patients had measurable levels at the thirty-sixth hour, and about one-third at the forty-eighth hour. The median levels at various hours are also shown in Table I.

While this study was in progress, several of the commercially available lots of procaine penicillin in oil were found to be inferior in that only about one-half to one-third of the patients had detectable levels in the blood at the twenty-fourth hour following the injection of 300,000 units of procaine penicillin in oil (1 c.c.).[8] It was found that in conversion to mass production, crystallization was not carefully controlled, so that large particles of procaine penicillin were

*Tween 80, Sorbitan mono-oleate, polypoxyalkylene derivative.

†Span 80, Sorbitan mono-oleate.

‡Supplied by Chas. Pfizer & Company, Inc., Brooklyn, N. Y., and Eli Lilly & Company, Indianapolis, Ind.

§Supplied by Bristol Laboratories, Inc., Syracuse, N. Y.

‖Supplied by Wyeth Incorporated, Philadelphia, Pa.

TABLE I. RESULTS FOLLOWING INTRAMUSCULAR ADMINISTRATION OF VARIOUS PREPARATIONS OF PROCAINE PENICILLIN (300,000 UNITS)

PREPARATION		HOUR										
		12	16	20	24	36	48	60	72	96	120	144
Procaine penicillin in oil	Median levels (U./c.c.)	0.375	0.375	0.25	0.125	0.031	0.031					
	Percentage of patients with assayable levels*	100	100	100	96	57	36					
Procaine penicillin (particles less than 5 μ) in oil plus aluminum monostearate	Median levels (U./c.c.)				0.25		0.125		0.062	0.062	0.031	0
	Percentage of patients with assayable levels*				100		100		100	100	100	42
Procaine penicillin plus sodium carboxymethylcellulose	Median levels (U./c.c.)	0.5	0.25	0.25	0.125	0.125	.062	0.062	0			
	Percentage of patients with assayable levels*	100	100	100	100	100	100	100	25			

At least twenty-five patients were studied at each time interval indicated for the various preparations.
*Method of Randall and associates[7] detecting 0.03 unit per cubic centimeter and higher.

produced. In grinding these particles to sizes capable of passage through 19 or 20 gauge needles, relatively large amounts of procaine penicillin dust or flour were produced. The fine particles comprising this dust or flour are dissolved and absorbed relatively rapidly, so that prolonged blood concentrations are not maintained. (The manufacturers have taken steps to eliminate the fine particles from their preparations.) Therefore, the blood concentrations obtained after the injection of preparations of this kind are not included in Table I.

The percentage of patients and the median blood concentrations following the injection of 300,000 units of procaine penicillin in oil plus 2 per cent W/V aluminum monostearate are also shown in Table I. It is apparent that the addition of aluminum monostearate not only stabilizes the suspension of procaine penicillin in oil but also results in prolongation of the concentrations of penicillin in the blood. Preparations containing particles of penicillin less than 5 μ in size resulted in measurable blood concentrations in all patients at twenty-four, forty-eight, seventy-two, ninety-six, and one hundred twenty hours after injection. When large particle procaine penicillin crystals are employed in this mixture, the concentrations of penicillin in the blood are not so prolonged.[4, 9]

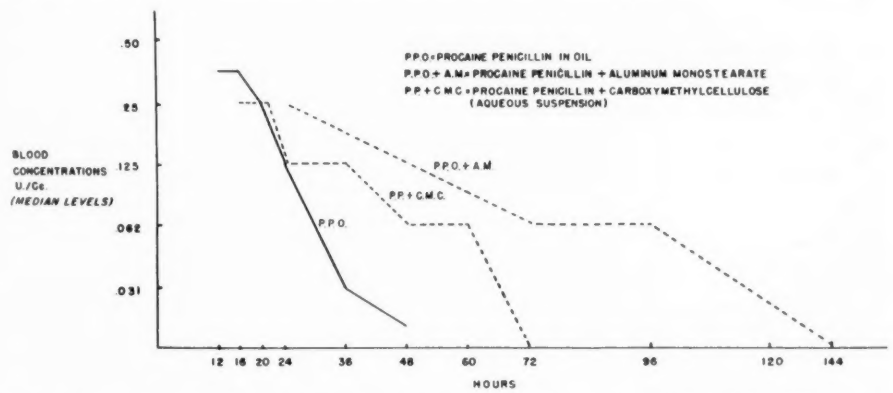

Fig. 1.—Curves of the concentration of penicillin in the blood following intramuscular injection of 300,000 units of procaine penicillin G suspended in various vehicles.

Included in Table I are the data obtained following the injection of 300,000 units of procaine penicillin plus 3.5 Gm. of sodium carboxymethylcellulose contained in 1 c.c. of aqueous solution. All of the patients studied had measurable levels at sixteen, twenty, twenty-four, thirty-six, forty-eight, and sixty hours. Twenty-five per cent had assayable levels at seventy-two hours.

The median concentrations at the various intervals for all three preparations have been plotted in Fig. 1.

Blood concentrations were determined in seventeen patients who were receiving 300,000 units of the procaine penicillin in oil preparations every twelve hours and in fourteen subjects who received 600,000 units (2 c.c.) every twelve hours. Upon such regimens the blood concentrations at the twelfth hour were at least 0.125 unit per cubic centimeter and usually 0.25 and 0.5 unit per cubic centimeter. There were no significant differences between the two doses.

CLINICAL STUDY

We have treated 251 patients with various infections with procaine penicillin in oil. All the patients received plain procaine penicillin in oil except the patients with gonorrhea who were treated with the material containing aluminum monostearate. The results and plans of therapy are summarized in Table II.

Eighty-nine patients with pneumococcic pneumonia of known type or with findings and a course characteristic of pneumococcic pneumonia were treated with 600,000 units of procaine penicillin in oil (2 c.c.) every twelve hours until they were essentially afebrile for forty-eight to seventy-two hours. The course was similar to that seen with the use of other penicillin preparations, and recovery was uneventful in all patients. These large doses were employed as a part of a study to evaluate the effect of massive doses in pneumonia. Other investigators[10] have found that 300,000 units a day for similar periods give satisfactory results.

TABLE II. DOSAGE SCHEDULES AND RESULTS OF TREATMENT OF VARIOUS INFECTIONS WITH PROCAINE PENICILLIN IN OIL

DISEASE	NUMBER OF PATIENTS	DOSAGE SCHEDULE	COMMENT
Pneumonia Typed 41 Untyped 48	89	600,000 units b.i.d. until essentially afebrile for 48 to 72 hours	Recovered
Acute bronchitis	2	300,000 to 600,000 units b.i.d. for 5 days	Recovered
Acute sinusitis	3	300,000 to 600,000 units b.i.d. for 5 days	Recovered
Tonsillitis	3	300,000 units per day for 5 days	Recovered
Scarlet fever	17	300,000 units per day for 5 days	Recovered
Vincent's infection	1	300,000 units per day for 2 days	Recovered
Infectious arthritis Gonococcal 2 Unknown 1	3	300,000 to 600,000 units b.i.d. for 7 to 10 days	Recovered
Gonorrhea*	57	300,000 units	Only 1 patient had return of symptoms—possible reinfection
Syphilis	75	600,000 units per day for 5 days	All patients showed complete healing of lesions and decrease in serologic titers during 2- to 5-month follow-up period
Cellulitis	1	600,000 units per day for 4 days	Recovered
Typhoid fever	1	300,000 units every 6 hours, with sulfathiazole—6 Gm. initial dose and 1 Gm. every 4 hours	No improvement

*Patients treated with procaine penicillin in oil plus aluminum monostearate.

In previous publications[11, 12, 13] the efficacy of penicillin in the treatment of scarlet fever has been reported. Procaine penicillin in oil in doses of 300,000 units per day for five days has resulted in prompt recovery from this streptococcal infection without pyogenic complications in all seventeen patients treated.

Three patients with bacterial arthritis were treated. Two were considered to have had gonococcal arthritis, since gonococci were isolated from a coexistent

urethral discharge. One of the patients was treated with 300,000 units of procaine penicillin in oil every twelve hours. Only slight improvement was noted after five days, at which time the dose was increased to 600,000 units every twelve hours and rapid improvement ensued. The second patient received 300,000 units every twelve hours for seven days with good results. These patients are included in a recent report.[14] The third patient was a young colored woman, six months pregnant, who had a profuse vaginal discharge and arthritis of the wrists. Gonococci were not isolated from cultures of the vaginal discharge. The patient was given 300,000 units of procaine penicillin in oil every twelve hours for seven days with rapid regression of the cervicitis and arthritis.

Two patients with acute bacterial bronchitis and three with acute sinusitis have been treated successfully. One of the patients with acute sinusitis also had acute catarrhal otitis media, the causative organism being a pneumococcus, Type 4. This patient was given 600,000 units (2 c.c.) of procaine penicillin in oil per day for four days. The temperature dropped to normal within twelve hours; the nasal discharge became less purulent and viscid, and was gone at the time treatment was discontinued.

A patient with Vincent's infection of the mouth received 300,000 units per day for three days. Relief from soreness was reported within twelve hours and the gums appeared normal at the forty-eighth hour.

Three patients with acute follicular tonsillitis were treated with 300,000 units of procaine penicillin in oil per day for five days. Pain on swallowing and soreness subsided rapidly, and the exudate promptly disappeared. A beta hemolytic streptococcus was isolated from the throat of one patient before treatment was started and was not found thereafter.

In the patient with typhoid fever, the bacteriologic diagnosis was made on the fourth hospital day. After several days, during which time there was no improvement on other therapy, it was decided to institute a routine similar to that used by Comerford and co-workers[15] in the treatment of typhoid carriers. Six grams of sulfathiazole were given initially, followed by 1 Gm. every four hours. In addition, 300,000 units of procaine penicillin in oil were given every six hours. The strain of typhoid bacillus which was recovered from the blood cultures was found to be resistant to more than 20 units per cubic centimeter of penicillin, whereas the blood penicillin concentrations prior to injection at the twelfth hour were found to be between 1 and 2 units per cubic centimeter of serum. There was no improvement after five days and therapy was discontinued. This patient developed an abscess on the buttock from which the typhoid organism was recovered. The source of this local infection was not established, although it is possible that the bacteria were carried in from the skin by the needles during injections of procaine penicillin. This patient later recovered on symptomatic treatment.

We have treated fifty-seven patients with acute gonorrhea using a single injection of 300,000 units of procaine penicillin in oil plus aluminum monostearate. The patients were followed for a period of twenty-one days. All the patients recovered except one who suffered a possible relapse during the third posttreatment week. It is believed, however, that this patient was re-exposed during the interim.

Seventy-five patients with primary and secondary syphilis were treated with 600,000 units (2 c.c.) per day for a period of five days. All the patients showed complete healing of lesions and a decrease in serologic titers during a follow-up period of two to five months.

REACTIONS

Four patients reported a slight degree of transitory soreness twelve to twenty-four hours after injection. We have given as much as 5 c.c. in a single injection to one patient without incident. A small area of induration with pruritis was observed in one patient at the site of injections ten days after the injections. Skin tests with the various ingredients of the preparation demonstrated sensitivity to peanut and sesame oils. We have not detected any other evidences of hypersensitivity to the preparation. It has been shown previously that when patients are given a second course of a drug, reactions are more likely to develop than during the initial course.[16, 17] Twenty-nine patients received a second course of procaine penicillin in oil without any reaction.

Doses of 300,000 units of procaine penicillin contain 120 mg. of procaine base per cubic centimeter. This amount of procaine base in the doses usually employed is not enough to produce toxic levels, particularly since the absorption into the circulation is slow and destruction is rapid. As much as 1 Gm. of procaine hydrochloride has been given intravenously over a period of twenty minutes without incident.[18] We have attempted to determine the procaine concentration in the blood, but the amounts present, if any, were so small as to make accurate colorimetric estimations impossible.

DISCUSSION

Procaine penicillin in oil as a means of prolonging therapeutic blood concentrations of penicillin appears to have certain advantages over penicillin in oil and wax. We have withdrawn and administered without difficulty procaine penicillin preparations with syringes sterilized by boiling which were damp when used. Procaine penicillin preparations maintain blood concentrations of penicillin which are adequate for most purposes. The best preparations of penicillin in oil and wax give detectable levels in 92 per cent of the patients with 300,000 units at the twenty-fourth hour, but the average preparation produces such levels in only about 85 per cent of patients. Similar doses of procaine penicillin in oil consistently result in adequate concentrations in at least 94 per cent of the patients and in 100 per cent of patients when optimal preparations are used. Not only do a greater percentage of patients have detectable concentrations at the twenty-fourth hour, but the concentrations are usually two- to fourfold higher than those obtained with penicillin in oil and wax. This is undoubtedly explained by the fact that the release of penicillin from the procaine combination is slow, resulting in a flat and sustained curve, while the one produced by the oil and wax preparation is peaked in the early hours and is not as well sustained. The problem of separation of the procaine penicillin from the oil has been overcome by the addition of aluminum monostearate. With the use of carboxymethylcellulose the procaine penicillin can be administered in aqueous suspension.

Another advantage of procaine penicillin preparations is the paucity of local reactions. Beeswax, which acts as a foreign substance and which may be antigenic, has been eliminated. We have not been able to demonstrate any sensitivity to procaine penicillin.

The maintenance of continued blood concentrations makes procaine penicillin in oil more desirable in the treatment of infections requiring short courses and more feasible in the cases requiring long courses. The results in the patients treated have been good. The only patients in whom no improvement followed the administration of procaine penicillin were the patient with typhoid fever and the one with acute gonorrhea who probably had a reinfection.

SUMMARY AND CONCLUSION

A new penicillin salt, procaine penicillin, incorporated in oil, in oil and aluminum monostearate, and with sodium carboxymethylcellulose in an aqueous solution has been studied.

The concentrations of penicillin in the blood at various intervals have been determined following intramuscular injections. The results are expressed both as percentage of patients having assayable levels and as the median levels at the various intervals tested.

Minimal local reactions and no systemic reactions were observed, even though twenty-nine patients received a second course.

Therapeutic results in patients with various infections were similar to those obtained with penicillin in other dosage forms.

It is concluded that procaine penicillin is a superior preparation for repository penicillin therapy since it does not require the use of dry syringes and needles, is followed by very few local reactions, and results in more prolonged blood penicillin concentrations than any penicillin preparation yet studied.

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ACQUIRED RESISTANCE OF PSITTACOSIS VIRUS TO SULFADIAZINE AND EFFECTS OF CHEMICAL ANTAGONISTS ON SULFONAMIDE ACTIVITY

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THE susceptibility of members of the psittacosis-lymphogranuloma group of viruses to sulfonamide activity has been shown to vary considerably, depending on the virus strain, the drug tested, and the host tissue employed.[1, 2] Among the viruses found to be susceptible to sulfadiazine was the 6BC strain of psittacosis virus[3-6] originally isolated by Dr. K. F. Meyer. It was shown that although chick embryos and mice could be protected from the lethal effect of the infection, active virus could be recovered from the tissues in most instances, indicating that growth was not completely inhibited. Jones, Rake, and Stearns[7] isolated lymphogranuloma venereum virus from mice treated with different sulfonamides and retested these strains in other mice maintained on a diet containing either sulfathiazole or sulfadiazine. Four out of eight strains appeared to show an increased resistance to the drug therapy.

It was considered of interest to investigate further the susceptibility of the 6BC strain of psittacosis virus to sulfadiazine, to test the effect of para-aminobenzoic acid (PABA) and pteroylglutamic acid† (PGA) on sulfonamide activity, and to develop a strain with increased resistance to sulfadiazine.

MATERIALS AND METHODS

The 6BC strain of psittacosis virus was used throughout this study. Yolk sac passages were maintained as 10 per cent suspensions by weight in nutrient broth, frozen and stored in a dry ice chest. From these stock preparations further tenfold dilutions were made in broth as desired, considering the 10 per cent suspension as a 10^{-1} dilution. Eight to nine day old embryonated eggs from White Leghorn chickens were used for inoculation into the yoke sac. LD_{50} estimations of virus activity were performed by the single dilution method[8] in which the average day of death of a group of eggs is used as the basis for the end point, employing previously determined standard curves for this strain of virus. Eggs were candled daily for ten days at twenty-four hour intervals from the time of inoculation. In many instances a delay in the average day of death was the only available criterion of drug effect, although if an effective drug was employed in higher concentrations, differences could be expressed in terms of per cent of surviving eggs.

Sodium sulfadiazine, sulfathiazole, sulfanilamide, sulfamerazine, and para-aminobenzoic acid were dissolved to the desired concentration in distilled water and sterilized by filtration through fritted glass filters. Just prior to inoculation the drug solution and the virus suspension in broth, pH 7.4, were mixed in the desired proportions. The pteroylglutamic acid was made up as its sodium salt in distilled water and inoculated into the eggs one-half hour before the virus.

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†The pteroylglutamic acid used was a commercial preparation, Folvite, manufactured by Lederle Laboratories.

RESULTS

Susceptibility of the Normal 6BC Strain of Psittacosis Virus to Sulfadiazine (SD).—Stock seed of 6BC yolk sac virus of known titer in eggs was diluted in broth to 10^{-3} through 10^{-6}. Equal volumes of these virus suspensions were mixed with solutions of sulfadiazine containing 0.5, 0.25, or 0.125 mg. per 0.1 ml. or with sterile distilled water as controls. Groups of twelve eggs were inoculated with 0.2 ml. of these mixtures by the yolk sac route. On the tenth day of the experiment the yolk sacs were harvested from four living eggs of each group receiving 0.5 mg. of sulfadiazine. To determine the virus concentration of the membranes at this period, each yolk sac pool was ground with sterile glass beads, diluted with sterile broth to a 10^{-2} dilution, and inoculated into thirty eggs. The results are shown in Table I.

TABLE I. SUSCEPTIBILITY OF THE 6BC STRAIN OF PSITTACOSIS VIRUS TO VARYING CONCENTRATIONS OF SULFADIAZINE

INOCULUM				
VIRUS (LD_{50} DOSES)	SULFADIAZINE (MG.)	D/T*	AVERAGE DAY OF DEATH	LD_{50} OF VIRUS FROM LIVING EGGS ON TENTH DAY†
$10^{4.8}$	0.5	1/12	9.0	$10^{-6.40}$
	0.25	11/12	8.0	------
	0.125	11/11	5.6	------
	None	10/10	4.2	------
$10^{3.8}$	0.5	2/10	9.5	$10^{-6.85}$
	0.25	8/ 9	8.1	------
	0.125	9/ 9	6.9	------
	None	11/11	5.1	------
$10^{2.8}$	0.5	0/10	--	$10^{-5.90}$
	0.25	7/11	8.3	------
	0.125	12/12	8.2	------
	None	12/12	5.4	------
$10^{1.8}$	0.5	1/12	10.0	$10^{-3.50}$
	0.25	4/10	9.5	------
	0.125	5/10	9.4	------
	None	11/11	5.9	------

*Dead mice per total inoculated.

†Pooled yolk sac material from four eggs of groups inoculated with 0.5 mg. of sulfadiazine.

A decrease in the concentration of the virus inoculum resulted in a more marked effect of the drug. However, even with the most concentrated virus inoculum, $10^{4.8}$ LD_{50} doses, a definite delay in death of the embryos was evident with 0.125 mg. of sulfadiazine, although the mortality was 100 per cent. The LD_{50} figures in the last column show that although many embryos were protected by 0.5 mg. of sulfadiazine, in each case active virus was recoverable from the yolk sacs in considerable amount.

Antagonistic Action of Para-Aminobenzoic Acid (PABA) and Pteroylglutamic Acid (PGA).—Para-aminobenzoic acid as a competitive antagonist of sulfonamides is well documented in experiments with bacteria.[9] Findlay[10] further reported an experiment in which the effect of sulfanilamide on lymphogranuloma venereum virus administered intracerebrally in mice was antagonized considerably by para-aminobenzoic acid in the diet. Subsequently, Seeler,

AVERAGE DAY OF DEATH OF EMBRYOS

Graessle, and Dusenbery[11] were unable to confirm Findlay's results and suggested that the mode of action of the sulfonamides on lymphogranuloma venereum virus differed from their action on other susceptible agents.

Pteroylglutamic acid also has been shown to antagonize sulfonamides, but this compound acts probably in a noncompetitive manner and is quantitatively less effective than para-aminobenzoic acid.[12, 13]

Shortly after the completion of our work, a report by Morgan[14] appeared in which antagonism of sulfadiazine inhibition of psittacosis virus by para-aminobenzoic acid and pteroylglutamic acid was reported. A competitive inhibition by para-aminobenzoic acid was indicated, whereas pteroylglutamic acid was said to be noncompetitive in action. As little as 0.05 mg. of pteroylglutamic acid was reported to be sufficient to antagonize 2.5 mg. of sulfadiazine in eggs infected with 10,000 LD_{50} doses. Apparently survival or death of the eggs during the ten-day observation period was the only criterion considered for antagonism of the sulfadiazine activity. From the tabulated results, 0.1 mg. of pteroylglutamic acid antagonized (all eggs dead) as much as 50 mg. of sulfadiazine. Our results are similar to those of Morgan with para-aminobenzoic acid but may differ somewhat in the experiments with pteroylglutamic acid, as will be seen from the data presented below.

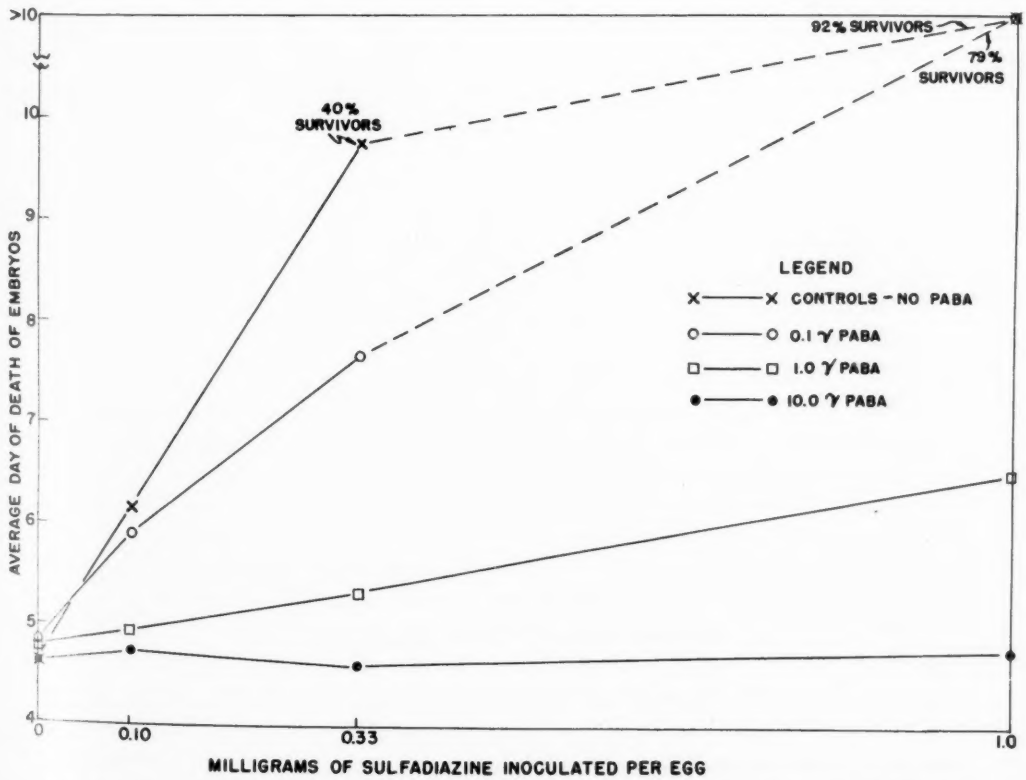

Fig. 1.—Antagonism of sulfadiazine by para-aminobenzoic acid with psittacosis virus in eggs.

Para-aminobenzoic acid was tested quantitatively for its antagonistic activity to sulfadiazine, employing the 6BC strain of psittacosis virus in eggs. The stock virus was diluted to 3×10^{-4} in M/15 phosphate buffer solution at pH 7.5. To aliquots of this virus suspension were added, just before inoculation, equal volumes of sulfadiazine solution (1, 0.33, or 0.1 mg. per 0.1 ml.) and para-aminobenzoic acid solution (10, 1.0, or 0.1 γ per 0.1 ml.). Distilled water was substituted for the chemicals in the control tubes. Each mixture was inoculated into thirty 8-day-old eggs by the yolk sac route, using 0.3 ml. per egg. The eggs were candled daily for the usual period of ten days. Fig. 1 shows the results obtained in terms of the average day of death of each group of eggs.

It can be seen by comparison of the values for the average day of death at the different sulfadiazine levels that complete or almost complete antagonism occurred with 10 γ of para-aminobenzoic acid against 1 mg. of sulfadiazine and with 1 γ of para-aminobenzoic acid against 0.1 mg. of sulfadiazine, which is evidence of competitive action. Even 0.1 γ of para-aminobenzoic acid manifested some antagonistic effect in the presence of 0.33 mg. of sulfadiazine; the average day of death was reduced from 9.7 in the control eggs to 7.6 in the test eggs. The relation between the concentration of sulfadiazine and the average day of death appeared to fall in a linear fashion within the ten-day observation period.

TABLE II. ANTAGONISM BY PTEROYLGLUTAMIC ACID OF SULFADIAZINE ACTIVITY AGAINST PSITTACOSIS VIRUS, 10,000 LD_{50} DOSE

PGA (MG.)	SD (MG.)	A.D.D.*	MORTALITY RATIO	PER CENT SURVIVORS
None	None	4.20	25/25	0
None	1	8.00	3/20	85
None	5	--	0/22	100
0.1	None	4.03	22/22	0
0.1	1	7.00	25/26	4
0.1	5	8.00	12/25	52
0.5	None	4.12	25/25	0
0.5	1	4.55	22/22	0
0.5	5	4.43	23/23	0

*Average day of death, living eggs not included.

Comparable experiments with pteroylglutamic acid as antagonist showed this compound to be less effective than para-aminobenzoic acid. Morgan's results, however, prompted investigation of higher concentrations than first tested by us. Accordingly, 0.1 and 0.5 mg. of pteroylglutamic acid, in the form of its sodium salt, were tested against 1 and 5 mg. of sulfadiazine in 7-day-old eggs, as used by Morgan. In order to duplicate conditions, the pteroylglutamic acid and sulfadiazine were inoculated as a mixture in a volume of 0.25 ml., followed by inoculation of approximately 10,000 LD_{50}. Results are shown in Table II in which the average day of death is listed along with the per cent survivors as an indicator of antagonism of the sulfadiazine activity.

It can be seen that 0.5 mg. of pteroylglutamic acid antagonized either 1 or 5 mg. of sulfadiazine to the extent that all the eggs died and the average days of death (4.43 and 4.55) closely approached the figure for the control group of

that series (4.12). Since it has been shown[8] that the growth rate of the virus in the yolk sac is mirrored in the average time of death, this measurement is considered to be a more delicate criterion for the degree of antagonism exerted. If virus growth is allowed to continue in a normal fashion, the average day of death of the test eggs will be very close to that of the control group and would indicate complete antagonism. With 0.1 mg. of pteroylglutamic acid, although considerable antagonism is evident against 1 mg. of sulfadiazine, where only one of twenty-six eggs survived, the average day of death was 7.00 as compared with 4.03 for the control. Against 5 mg. of sulfadiazine approximately half of the inoculated eggs survived and the remainder showed an average day of death of 8.00. These results indicate that the antagonism by pteroylglutamic acid is not complete unless heavy concentrations are employed. When 0.1 mg. of pteroylglutamic acid was tested against 50 mg. of sulfadiazine, eleven out of eighteen eggs survived, and the eggs that died had an average day of death of 8.30 compared with 4.23 for the controls. It is possible that free para-aminobenzoic acid as an impurity in the pteroylglutamic acid preparations may be a factor in the activity shown.

It should be mentioned, also, that in every instance in which pteroylglutamic acid was inoculated with the virus in the absence of sulfadiazine, the average day of death of the eggs was slightly lower than that of the controls. Although the differences were small, between 0.1 and 0.5 days, they were consistent and suggest a slight stimulating effect of this compound on the growth of the virus.

Since the reports in the literature[10, 11] concerning the antagonism of para-aminobenzoic acid to sulfonamides with lymphogranuloma venereum were contradictory, an attempt was made to test the effect of this antagonist against sulfadiazine using lymphogranuloma venereum virus in embryonated eggs. Stock lymphogranuloma venereum virus of yolk sac origin was diluted to 10^{-2}. Equal volume mixtures of the virus suspension with sulfadiazine solution (0.2 mg. per 0.1 ml.) and/or para-aminobenzoic acid solution (0.01 mg. per 0.1 ml.) were prepared and inoculated into groups of thirty 8-day-old embryonated eggs by the yolk sac route. Distilled water was substituted in the control tubes. The eggs were candled daily for ten days.

Although all the eggs inoculated with the control preparations, that is, virus alone or virus with para-aminobenzoic acid, were killed, none died of those receiving virus plus sulfadiazine, and only two of twenty-seven eggs died of those receiving both sulfadiazine and para-aminobenzoic acid with the virus. Further, duplicate yolk sac pools of living eggs collected from each group on the tenth day of incubation did not indicate any significant differences in virus content between those eggs receiving an inoculum of sulfadiazine alone and those receiving sulfadiazine with para-aminobenzoic acid. Thus it is apparent that the para-aminobenzoic acid did not antagonize the action of the sulfadiazine as it did when psittacosis virus was employed as the test agent. These results tend to confirm the negative findings of Seeler and co-workers[11] with this agent, with the mouse as test animal.

Development of a Sulfadiazine-Resistant Strain of Psittacosis Virus.—In order to develop a resistant strain, the stock seed of 6BC virus, diluted usually

to 10^{-3}, was passed five times by the allantoic route in the presence of 0.5 mg. of sulfadiazine. Very few of the eggs thus inoculated died during the ten-day observation period, so allantoic fluid from living eggs was harvested for passage on the third to the eighth day. Little or no increase in resistance to the sulfadiazine was noted during this period upon test either by the allantoic or yolk sac route. It was again shown, however, that the allantoic fluid from living eggs inoculated with virus and sulfadiazine harbored active virus, often in considerable concentration, titrating to LD_{50} values as high as $10^{-7.3}$. Yolk sac material was harvested from five eggs, dead on the tenth day, which had received an inoculum of virus from the fifth sulfadiazine passage, plus 0.5 mg. of sulfadiazine. This passage virus, 6BC-SD-6, when compared with the parent virus in the presence of 0.5 or 0.25 mg. of sulfadiazine, showed some increased resistance as manifested by a reduction in the average day of death. When the daily growth of this passage virus in the yolk sac was compared with that of the parent strain in the presence and absence of 0.5 mg. of sulfadiazine, the curve of the 6BC-SD-6 strain lay higher than that of the normal strain in the presence of sulfadiazine, but lower than in its absence. This indicated only partial resistance of this passage virus to the drug. This strain was then passed four more times in a 10^{-2} dilution by the yolk sac route in the presence of 0.5 mg. of sulfadiazine, employing yolk sac material harvested from dead eggs on the third to the fifth day. The final harvest, having been passed a total of ten times in the presence of this drug, was labelled 6BC-SD-10.

The LD_{50} titer of this virus in eggs by the yolk sac route was $10^{-8.20}$. The growth curves of this strain were compared with those of a parent stock strain of the same initial titer. Each virus was diluted to a 10^{-3} dilution in broth and mixed with an equal volume of sulfadiazine solution containing 0.5 mg. per 0.1 ml. or distilled water as a control. Groups of 8-day-old eggs were inoculated by the yolk sac route with 0.2 ml., and at intervals of four hours and of one, three, four, and six days yolk sac pools were harvested from four live eggs of each group as long as they were available. These pools were then each titrated by the single dilution method in twenty-four eggs. The results are shown in Fig. 2.

It is apparent that the growth of the sulfadiazine-resistant strain of virus, 6BC-SD-10, in the presence of 0.5 mg. of the drug was practically identical with that of the same strain or the stock strain in the absence of sulfadiazine. All of these eggs were dead by the fifth day. On the other hand, the stock strain in the presence of the sulfadiazine showed definitely slower growth and, among a group of thirty separate eggs of this series, there were 41 per cent survivors at the end of ten days. This strain was further shown to have developed complete resistance to 0.2 mg. amounts of sulfathiazole and sulfamerazine. Sulfanilamide, in the same concentration, had no significant effect on the parent strain, so that no difference was detectable with this compound.

Following transfer of the 6BC-SD-10 strain through ten rapid passages by the yolk sac route in eggs in the absence of sulfadiazine, the titer in the yolk sacs reached approximately 10^{-10}. When retested for its activity in the presence of 0.5 mg. of sulfadiazine, it was found to have retained complete resistance to

this concentration of the drug. It was then tested against concentrations of sulfadiazine as high as 20 mg. per egg, and complete resistance was still evident. Since considerable virus of a normal strain is enabled to grow out in the presence of sulfonamides, it is possible that selective breeding plays an important role in the development of a highly resistant progeny by repeated passage.

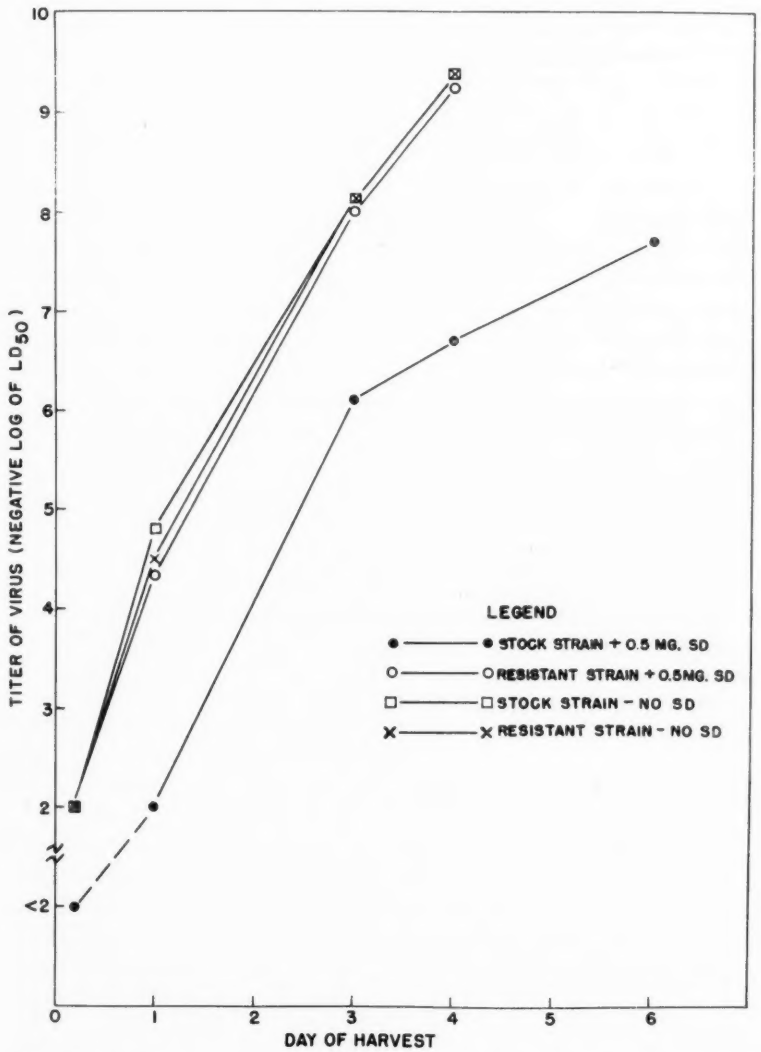

Fig. 2.—Growth curves of stock strain and resistant strain of psittacosis virus in the presence and absence of sulfadiazine.

An allantoic fluid preparation of the resistant strain was partially purified by several cycles of centrifugation and examined under the electron microscope. No significant differences in morphology were found to distinguish it from similar preparations of the parent virus.

SUMMARY

The 6BC strain of psittacosis virus, although quite susceptible to the action of sulfadiazine in eggs as shown by reduced mortality, multiplied to a considerable extent in the presence of the drug.

Para-aminobenzoic acid was found to be a highly effective antagonist of sulfadiazine with this virus in eggs, and a competitive relationship was suggested. A similar test with the virus of lymphogranuloma venereum did not show any antagonistic effect.

Pteroylglutamic acid was found to require considerably higher concentrations for demonstration of complete antagonism against as little as 1 mg. of sulfadiazine, although partial antagonism was demonstrable against 50 mg. of sulfadiazine.

By repeated passage of the 6BC virus in the presence of sulfadiazine, a strain was developed which was completely resistant to 20 mg. of the drug even after ten passages through normal eggs. Concomitant resistance of this strain to sulfathiazole and sulfamerazine was also demonstrated. No morphologic differences between the resistant strain and the parent virus were observed with the electron microscope.

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THE EFFECT OF NICOTINIC ACID AMIDE ON EXPERIMENTAL TUBERCULOSIS OF WHITE MICE

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INTRODUCTION

IN SCREENING some two to three thousand chemicals against the tubercle bacillus on the chorioallantoic membrane of the chick embryo, we[1] found that 15 per cent of the compounds were inhibitory. Later all of these active chemicals were screened according to a technique that utilizes white mice. This technique was introduced by two Russian workers, Shpanier and Chertkova,[2] in 1944 and by Youmans and McCarter[3] in this country in 1945.

In the Russian report the mice were infected by an intravenous injection of 0.5 mg. of a 2-week-old culture of the human type of tubercle bacillus, $H_{37}Rv$. They were treated with intramuscular injections of the chemical suspended in oil, receiving ten such injections in fifteen days. Evaluation of the results was made by culturing a suspension of the parenchymal organs on egg medium and after one month examining for macroscopic growth of the tubercle bacillus.

In this country Youmans and McCarter[3] developed the technique of producing experimental tuberculosis in mice and established a readily reproducible test. Thus they contributed a convenient in vivo method of screening a large number of compounds. They injected intravenously 0.1 mg. of a T.B. suspension made from a 3-week-old culture. Youmans[4, 5] used this technique with considerable success in demonstrating the activity of streptomycin and later the activity of *para*-amino-salicylic acid.

EXPERIMENTAL

Swiss mice* weighing approximately 17 to 20 grams were used in our experiments. These were infected intravenously with 0.25 mg. of a T.B. suspension ($H_{37}Rv$) made from a 14- to 18-day-old culture. Animals so infected and left untreated usually died in three to three and one-half weeks. Though there was no apparent loss of weight in the first one to two weeks, the animals lost weight in the latter stage of infection, and at the time of death they usually weighed 15 to 18 grams. For the most part, oral treatment of infected animals was begun on the day after inoculation. The compounds were administered in the diet. The diet consisted of ground Rockland mouse pellets.

Nature of Infection.—Though the bacteria were given intravenously, there was no generalized miliary tuberculosis. Instead the disease seemed to be centered mainly in the lungs; the other organs such as the liver, spleen, and

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*Obtained from Tumblebrook Farm, Brant Lake, N. Y.

TABLE I. TREATMENT OF EXPERIMENTAL TUBERCULOSIS OF WHITE MICE WITH DERIVATIVES OF NICOTINIC ACID

CHEMICAL NUMBER	CHEMICAL NAME	PER CENT CHEMICAL IN DIET	AVERAGE BEGINNING WEIGHTS (GM.)	AVERAGE WEIGHTS (3 WK.)	PER CENT SURVIVAL	GROSS APPEARANCE OF EXCISED LUNG‡	AMOUNT OF DISEASE SHOWN IN LUNG SECTION‡	ACTIVITY OF TREATMENT CHEMICAL§
	None (uninfected mice)	None	19.4	22.6	100	0	0	
A217N	Streptomycin*	None	19.8	20.1	100	0	±1	Active
606L	Nicotinic acid amide	0.75	18.4	21.0	90	1	1	Active
		0.5	18.4	22.6	100	1	2	Sl. Active
525L	*N*-Nicotinyl-3-aminopyridine	0.5	19.0	16.1	70	2-3	2-3	V. Sl. Active
526L	*N*-(2-Thiazolyl)nicotinamide	0.12†	19.1	17.9	60	2-3	2	V. Sl. Active
528L	*N*-(2-Pyrimidyl)nicotinamide	1.5	19.0	16.2	40	2	2-3	V. Sl. Active
738L	*N*-Isopropylnicotinamide	0.25	18.8	16.1	10	2-3	2	V. Sl. Active
732L	*N*-Butylnicotinamide	0.25	19.8	19.0	10	2-3	2-3	V. Sl. Active
644L	*N*-γ-Piperidyl-propyl-nicotinamide	0.25	19.0	19.5	50	2	2-3	V. Sl. Active
668L	*N*-Nicotinoyl derivative of impure Merfanil	0.25	19.5	17.0	0	3	3	V. Sl. Active
637L	*N*-Nicotinyl-1-aminoanthraquinone	0.25	20.8	19.3	100	2	3	None
638L	*N*-Cyclohexylnicotinamide	0.25	19.3	17.6	10	4	4	None
640L	*N*-Dodecylnicotinamide	0.25†	19.6	17.0	20	4	4	None
642L	5-(Nicotinylamino)-2-methyl-coumarane	0.25	19.0	19.0	20	4	4	None
650L	Nicotinyldicyandiamide	0.25	20.1	19.0	50	4	4	None
641L	*N*-Nicotinylbenzylamine	0.25	20.1	15.0	0	4	4	None
645L	4-(Nicotinylamino)-salicylic acid	0.25	20.5	20.2	40	4	4	None
527L	*N*-Nicotinyl-2-aminopyridine	1.5†	19.0	17.0	10	4	4	None
649L	*N*-Nicotinyl-2-amino-5-azoanisole	0.25	20.2	20.3	50	4	4	None
652L	2-(Nicotinylamino)phenol	0.25	17.7	20.5	10	4	4	None
653L	3-(Nicotinylamino)phenol	0.25	17.8	19.7	10	4	4	None
654L	4-(Nicotinylamino)phenol	0.25	17.8	20.2	10	4	4	None
662L	d,l-α-Nicotinylaminobutyric acid	0.25	20.6	17.1	10	4	4	None
729L	*p*-(Nicotinylamino)benzoic acid	0.25	20.0	20.3	40	4	4	None
725L	2-(*N*-Nicotinylamino)-5-carbethoxy-thiazole	0.25	18.5	15.6	0	4	4	None
739L	*N*-Methoxypropylnicotinamide	0.25	18.5	16.4	10	4	4	None
661L	*p*-(Nicotinylamino)acetanilide	0.25	20.4	18	20	4	4	None
747L	6-Chloronicotinamide	0.25	18.5	13.5	10	4	4	None
765L or 770L	6-Aminonicotinamide	0.1†	19.4					
768L	6-Butoxynicotinamide	0.25	20.1	16.1	10	4	4	None
724L	2-Aminonicotinamide	0.25	19.8	16.5	0	4	4	None
749L	Ethyl-nicotinate	0.25	19.0	18.5	10	4	4	None

All treatment chemicals were included in the diet at the maximum tolerated concentration.

*Instead of being administered in the diet, streptomycin was given subcutaneously, 1 mg. four times daily.

†Treatment concentration is toxic.

‡0, No gross lesions; no disease in the lungs. ±, No gross lesions; microscopically, very slight amount of monocytic exudate. 4, Maximum amount of pathology observed: grossly, necrotic tubercles; microscopically, caseation and numerous acid fast bacilli. 3, 75 per cent of the maximum amount of pathology; grossly, gray necrotic areas; microscopically, small amount of caseation to massive monocytic and lymphocytic infiltration and clumps of acid-fast bacilli. 2, 50 per cent of the maximum amount of pathology; grossly, a very few necrotic areas; microscopically, sparse discrete areas of monocytic exudate. 1, 25 per cent of the maximum pathology; grossly, marked enlargement and congestion; microscopically, predominately a clear lung—a few acid-fast bacilli present.

§Some gross appearance as untreated streptomycin treated mice. Active, No gross appearance of pathology; microscopically a clear lung

lymph nodes were but slightly involved. The infection in the lungs at the time of death was extensive, varying from discrete white nodular patches to extreme consolidation of whole lobes. Microscopically these nodular patches showed varying stages of consolidation, caseation, and monocytic and lymphocytic infiltration to a mere proliferation of tissue. Numerous clumps of acid-fast bacilli were observed throughout the tubercles.

Chemotherapy.—The apparent tuberculostatic activity of pyridine carboxylic acid on the chorioallantoic membrane of the chick embryo led us to repeat all of the active compounds in infected mice. For the most part, these compounds were fed in the diet. Of this group nicotinic acid and its amide seemed to be the most active compounds. Consequently, Kushner and coworkers[6] synthesized a series of thirty derivatives of nicotinamide either in the form of substituents in the acid amide group or as nuclear substitutions. These were tested in mice by the technique already described. The results are given in Table I, but the chemical synthesis and properties of these compounds are published elsewhere.[6] Nicotinamide was the most active chemical tested. All the changes introduced into the nicotinic acid amide molecule either increased the toxicity or reduced the activity or both.

The failure to increase the activity or even to retain the activity of nicotinic acid amide by any slight alteration in the molecule led us to suspect that we might be dealing with the specific activity of a vitamin. Calculating from our results with mice, on a weight basis the therapeutic dose in human beings would be about 100 to 125 grams—a dose which could not be tolerated by the human subject. So other vitamins were tried at 0.1 per cent concentration in the diet with or without a smaller amount of nicotinic acid amide. Of these, only riboflavin seemed to have a slight effect in securing the desired end of reducing the total treatment dose of the nicotinic acid amid. See Table II.

In addition, possible naturally occurring precursors of nicotinic acid amide, such as tryptophane and 3-hydroxy-anthranilic acid, were attempted as chemotherapy, but all yielded negative results.

Resistance of the Tubercle Bacilli to Nicotinic Acid Amide.—One of the possible disadvantages of streptomycin in the treatment of human tuberculosis is that the organisms become resistant to streptomycin in the course of time. Similar resistance studies were made in regard to the possibility of the organisms' becoming resistant to the nicotinic acid amide, The organisms were recovered from the mouse previously treated with nicotinic acid amide and cultured on egg medium. The bacterial growth was resuspended and used for infecting the test mice. Five mouse passages showed little evidence of resistance of the tubercle bacilli, but later passages showed some evidence of resistance.

However, when the suspension of the lung excised from the mouse that had been treated with a combination of nicotinic acid amide and streptomycin was placed on egg medium, such a sparse growth appeared after a prolonged period of incubation that we believe a very much more reduced number of viable organisms existed in the lungs than when streptomycin was administered alone.

TABLE II. EFFECT OF TREATING EXPERIMENTAL TUBERCULOSIS OF WHITE MICE WITH NICOTINIC ACID AMIDE IN COMBINATION WITH DIFFERENT VITAMINS

VITAMIN	PER CENT NICOTINIC ACID AMIDE IN DIET	PER CENT ADDITIONAL VITAMIN IN DIET	AVERAGE BEGINNING WEIGHTS (GM.)	AVERAGE WEIGHTS (3 WK.)	PER CENT SURVIVAL (10 MICE PER GROUP)	GROSS APPEARANCE OF LUNG AT AUTOPSY†	AMOUNT OF DISEASE IN LUNG SECTION†	ACTIVITY‡
None	None	None	17.6	24.1	100	0		
Streptomycin*	None	None	17.8	23.2	100	0	±-1	Active
Streptomycin* plus nicotinic acid amide	0.75		19.2	21.6	100	0	±-1	Active
Nicotinic acid amide	0.25	None	18.4	20.9	80	2	2-3	V. Sl. Active
Nicotinic acid amide	0.5	None	18.4	22.6	100	1	2	Sl. Active
Riboflavin	None	1	19.0	18.8	0	4	4	None
Nicotinic acid amide plus riboflavin	0.25	0.1	20.2	20.9	50	2	2-3	V. Sl. Active
Nicotinic acid amide plus riboflavin	0.25	0.5	19.2	21.2	100	0	1-2	Active
Nicotinic acid amide plus calcium pantothenate	0.1	0.1	19.7	17.9	0	3-4	4	
Nicotinic acid amide plus calcium pantothenate	0.25	0.5	19.0	20.8	30	4	4	None
Nicotinic acid amide plus para-aminobenzoic acid	0.1	0.1	19.7	18.7	0	4	4	None
Nicotinic acid amide plus para-aminobenzoic acid	0.25	0.1	19.7	18.9	30	4	4	None
Nicotinic acid amide plus inositol	0.1	0.1	18.8	17.9	20	4	4	None
Nicotinic acid amide plus inositol	0.25	0.1	18.8	17.0	10	4	4	None
Nicotinic acid amide plus choline chloride	0.1	0.1	18.9	16.0	20	4	4	None
Nicotinic acid amide plus choline chloride	0.25	0.1	18.8	17.6	30	4	4	None
Nicotinic acid amide plus ascorbic acid	0.1	0.1	20.4	20.3	30	4	4	None
Nicotinic acid amide plus ascorbic acid	0.25	0.1	20.4	20.5	80	4	4	None
Nicotinic acid amide plus mixed tocopherol	0.25	0.1	18.4	16.4	0	4	4	None
Nicotinic acid amide plus vitamins A and D	0.25	A: 450 u./day D: 108 u./day	18.6	16.2	0	4	4	None
Nicotinic acid amide plus vitamins A and D	0.1	A: 450 u./day D: 108 u./day	18.9	15.2	0	4	4	None
None (untreated tuberculous mice)			19.2	15.6	0	4	4	

All treatment chemicals were included in the diet at the maximum tolerated concentration.

*See footnote *, Table I.

†See footnote ‡, Table I.

‡See footnote §, Table I.

SUMMARY

The oral administration of 0.5 to 0.75 per cent of nicotinic acid amide in the diet will markedly suppress the spread of tuberculosis in experimentally infected mice.

This amount of nicotinic acid amide in the diet was apparently roughly equivalent in activity to injections of 1 mg. of streptomycin four times daily over the same test period.

We wish to express our appreciation to Miss Florence Anderson, Miss Barbara Gosford, Mr. Samuel Smith, and Miss Hester Snider for their technical assistance.

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‡See footnote §, Table I.

PORPHOBILINOGEN TESTS ON A THOUSAND MISCELLANEOUS PATIENTS IN A SEARCH FOR FALSE POSITIVE REACTIONS

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IN THE last decade acute idiopathic porphyria has been accorded increasing attention as evidenced by more frequent clinical reports of the disease, reviews of the subject, and the description[8] of a new simple diagnostic laboratory procedure. It seemed pertinent to study the Watson and Schwartz porphobilinogen test[8] which is now widely considered as pathognomonic of this disease. The studies reported in this paper consisted of a search for false positive porphobilinogen tests in a large series of patients.

Briefly, porphyria is defined as a primary idiopathic disease, characterized by abnormal amounts of uro- and coproporphyrins and/or porphobilinogen in the urine, due to a derangement of the metabolism of porphyrin compounds. The term porphyria is opposed to porphyrinuria, which refers to abnormal amounts of coproporphyrins in the urine secondary to a variety of diseases such as lead poisoning, liver disease, or the ingestion of drugs (for instance sulfa drugs, Pyramidon or the salicylates). Porphyria is usually divided into two types: the congenital (or light-sensitive) and the acute idiopathic. Congenital porphyria is the rarest type and is characterized by red porphyrin deposits in the bones and teeth (erythrodontia), photosensitivity of the skin (hydroa aestivale sen vacciniforme), and a dark-red urine. Acute porphyria occurs in adults with intermittent attacks of acute abdominal crises with cramps and constipation, nerve paralysis and mental symptoms, and frequently a dark urine. The porphobilinogen test (see below) is characteristically positive, while in a small series of cases of congenital porphyria, the porphobilinogen test has been negative according to Watson, Schwartz and Hawkinson.[9]

For years the diagnosis of acute porphyria hinged on the large amounts of uro- and coproporphyrin found in the urine. The excretion of the Waldenström type of uroporphyrin in particular was shown to be increased in acute idiopathic porphyria.[5, 9] For a long time the diagnosis of porphyria depended on the chemical identification of uroporphyrin and, to a lesser extent, coproporphyrin, which required a difficult and tedious fractionation. The porphobilinogen test[8] is a simple procedure useful in the diagnosis of acute idiopathic porphyria.

HISTORY AND PROPERTIES OF PORPHOBILINOGEN AND PORPHOBILIN

In 1931 Sachs[4] first reported evidence of a substance in the urine of a patient with acute porphyria which gave a red color with Ehrlich's reagent (p-dimethyl-amidobenzaldehyde). She reported that this red compound exhibited two spectral absorption bands with Ehrlich's reagent. The red Ehrlich

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aldehyde compound was insoluble in chloroform, differentiating it from the urobilinogen-aldehyde compound and, as shown later by Watson and Schwartz,[9] from indole-aldehyde compound, as formed with Ehrlich's reagent.

Waldenström and Vahlquist discovered further properties of this substance.[5, 6, 7] They found that if the urine in acute porphyria was exposed to sunlight it became darker and the reaction with Ehrlich's reagent became negative. Urea inhibited the development of the reaction. Heating the acute porphyria urine with acid caused darkening concomitant with development of a negative Ehrlich's reaction and the appearance of a dark reddish-brown pigment which was named porphobilin.[6] Some evidence was elicited by Waldenström that this pigment was a dipyrrylmethene.

The colorless chromogen of this pigment was designated by Waldenström as porphobilinogen. A method for measuring porphobilinogen in arbitrary units has been described.[6] Neither porphobilinogen nor porphobilin has been chemically isolated or structurally identified. Prunty has studied the chemical properties of porphobilinogen and has isolated it from the liver in a patient with acute porphyria.[3] This work indicates that it is not a breakdown product in the urine, but is formed in the body, probably in the liver. Further studies are necessary to evaluate its possible role as a precursor of the porphyrin ring.

Watson and Schwartz[8] provided the first simple clinical test for acute idiopathic porphyria in 1941 and found it to be positive in all of five cases. Others have added confirmatory evidence that the test is at least relatively specific.[1, 2, 3]

Watson states[10] that in his laboratories several instances of a false red or pink color have occurred in a porphobilinogen test. One was a case of acute poliomyelitis in which the urine contained large amounts of coproporphyrin and urobilinogen. In a case of cirrhosis of the liver with large amounts of coproporphyrin in the urine, the porphobilinogen reaction was repeatedly, although not consistently, positive. Several urine specimens were found with large amounts of urobilinogen in which it was impossible with repeated extractions to get all of the aldehyde compound into the chloroform. The urine from a patient with jaundice showed a positive porphobilinogen test in which the red color behaved like an indicator with variations of pH, the red color disappearing with alkalinization. In this instance the red color was thought to be due to an extrinsic pigment, perhaps a drug. Crayons and beets ingested by infants gave weak but definitely positive porphobilinogen reactions. Watson now considers the test "in the main very helpful and reliable for porphyria, but I do not believe the test can be spoken of as absolutely specific or pathognomonic."

METHODS AND MATERIALS

The purpose of the present study was to evaluate this simple office procedure in regard to false reactions. There is no conclusive method for ruling out false positive reactions with any simple test except by means of large-scale examination of patients with miscellaneous diseases. The tests were done in the early morning with fresh urine, and from ten to thirty-five were done each day. Urine specimens of 1,000 different patients were examined by the porphobilinogen test of Watson and Schwartz in which 1 ml. of Ehrlich's reagent (0.7 Gm.

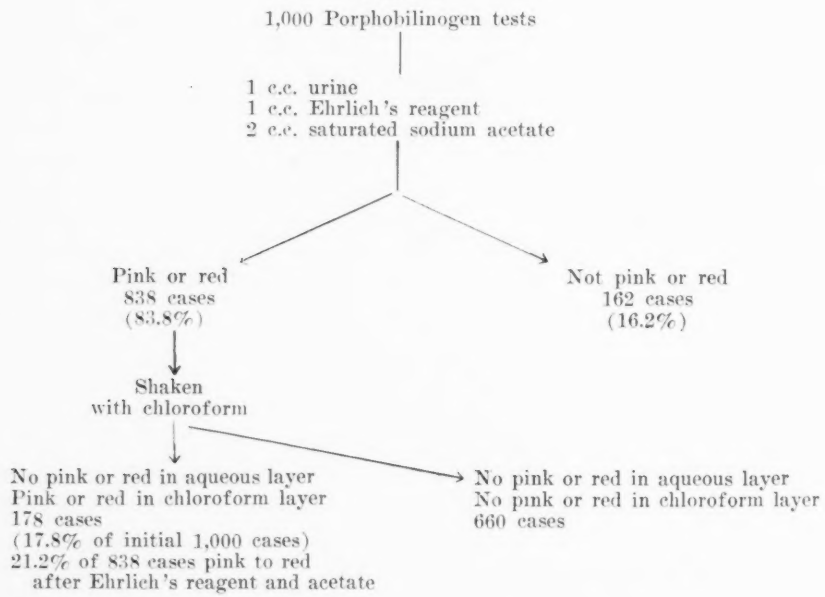
p-dimethyl-amidobenzaldehyde, 150 ml. concentrated HCl, and 100 ml. distilled water) was mixed with 1 ml. of urine, and then 2 ml. of a saturated aqueous solution of chemically pure sodium acetate also were added and mixed. If any pinkish or reddish color resulted, a few milliliters of chloroform were added and the whole was forcibly shaken in a stoppered tube.

The 1,000 patients on which the study was based came from the medical, surgical, obstetric, gynecologic, urologic, orthopedic, neurological, eye, ear, nose, and throat services of the Worcester City Hospital.

RESULTS

No positive porphobilinogen tests were obtained. In no case did a pinkish or red color remain in the aqueous layer when the test was performed properly. It is important to call attention to three points in particular: (1) The chloroform extraction requires a very thorough shaking with the aqueous fraction. (2) Red-colored globules of chloroform frequently adhere to the sides of the

CHART I



tube in the upper portion giving a false color to the aqueous layer. (3) Reflection of the reddish color from the lower (chloroform) layer into the aqueous layer must be discounted. The authors have been consulted by clinicians who were confused by what purported to be a positive porphobilinogen test in which one of the errors mentioned had occurred. The necessity for these precautions is emphasized by the fact that 838 (83.8 per cent) of the 1,000 cases showed some pink color in the aqueous layer prior to the addition of chloroform. In the majority of the 838 cases the color was a faint pink and in these all that remained after the addition of chloroform was a yellow or slightly orange tint in the chloroform layer. In 21 per cent of the 838 cases the chloroform layer became pink or red after the extraction procedure. The distribution of the reactions is represented by Chart I.

SUMMARY

The simple porphobilinogen test as described by Watson and Schwartz was studied by the examination of the urine of 1,000 patients.

No positive reactions were found. From this it may be concluded that false positive reactions are extremely rare, and that the test has a great deal of significance in the diagnosis of porphyria.

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HEPATIC DYSFUNCTION IN INFECTIOUS MONONUCLEOSIS; WITH REVIEW OF THE LITERATURE

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THE following paper is a report of the incidence of hepatic dysfunction in a group of hospitalized patients with infectious mononucleosis, with a review of the literature on the subject of liver involvement in infectious mononucleosis.

REVIEW OF LITERATURE

Pfeiffer,[1] in his original description of the disease called glandular fever, mentioned enlargement of the liver as one of the physical findings. Snapper and co-workers[2] were the first to describe jaundice as a complication of glandular fever. Downey and McKinlay[3] in 1923 were the first observers in this country to describe jaundice in a patient with infectious mononucleosis. Later, in 1935, McKinlay[4] reported a series of fifty cases with jaundice in 10 per cent. McKay and Wakefield[5] in 1926 reported one case of infectious mononucleosis with jaundice. Schmidheimy[6] in 1927 reported on eleven cases, with icterus in four. Mason[7] in 1928 reported two cases with jaundice and suggested that the jaundice was the result of a hepatitis, rather than the result of pressure of enlarged nodes on the common bile duct as had been suggested by McKay and Wakefield.[5]

Nyfeldt[8] reported a series of thirty-three cases with hepatomegaly in nine and icterus in four, and in 1934 Stuart and co-workers[9] reported a series of twenty-eight cases with jaundice in two. Farley[10] reported twelve cases with hepatomegaly in four, clinical jaundice in one, and bilirubinuria in an additional one. DeVries[11] reported three cases with clinical jaundice in 1938. Paul,[12] in a review of infectious mononucleosis in 1939, reported an incidence of jaundice in 10 per cent of fifty cases. In a later paper Gardner and Paul[13] reviewed 137 cases at the New Haven Hospital and found jaundice in only five per cent; however, 13 per cent had hepatomegaly.

Svaar-Seljesaeter,[14] Fowler and Tidrick,[15] Bernstein,[16] Gold,[17] Howard,[18] Carter,[19] Boger,[20] Monat,[21] Leavell and McNeal,[22] and Wising[23] each described one case with jaundice. Martin[24] reported two cases with jaundice, and Chapman and Chapman[25] reported seven cases with mild clinical jaundice.

Ollgaard[26] reported a large series of 210 cases with icterus in four. Ottenberg and Spieegel[27] reported sixty-seven cases with jaundice in two; Contratto,[28] 196 cases with ten jaundiced; Immerman,[29] 220 cases with three jaundiced; Milne,[30] 111 cases with three jaundiced; Read and Helweg,[31] 300 cases with jaundice in eleven but with forty-seven showing hepatomegaly. Spring[32] described the clinical picture in five cases with an elevated icteric index. Press

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and co-workers[33] reported on a series of ninety-six cases with jaundice as the presenting complaint in five, though the liver was enlarged in twenty-six of the patients.

Recently Abrams[34] reported one case with jaundice of eleven weeks' duration. He also mentions an incidence of jaundice of 9.4 per cent in a series of sixty-four cases.

Halcrow and associates,[35] in a report of an epidemic of infectious mononucleosis in a hospital in England, found no clinical jaundice in 296 cases. However, only 125 of the patients had clinical symptoms, whereas the other 165 had only blood and/or serologic changes. They studied fifteen of the more severe cases selected at random and found latent jaundice, as manifested by a slightly elevated serum bilirubin, in eight.

In a recent report of an epidemic extending over a fifteen-month period at an Army post, Wechsler and Rosenblum,[36] in the largest series of cases of infectious mononucleosis ever reported (556 cases), found thirty-five cases (7.5 per cent) with jaundice. Most of the cases were mild as far as the icteric index and gastrointestinal symptoms were concerned. They found that the liver was palpable in ninety-five of the 556 cases; however, they did liver function tests only in those that showed clinical jaundice. Liver function tests in many of these patients showed an elevated direct reacting bilirubin, positive cephalin-cholesterol test, elevated serum alkaline phosphatase, increased bromsulfalein retention, and elevated urine urobilinogen. In none of the patients that showed jaundice were there any sequelae.

Cohn and Lidman[37] reported on a group of fifteen successive patients hospitalized at an Army hospital for infectious mononucleosis. None of the fifteen were jaundiced clinically; however, by means of serial hepatic function studies they were able to demonstrate evidence of hepatic dysfunction in all fifteen. DeMarsh and Alt[38] reported nineteen consecutive cases of infectious mononucleosis without jaundice (icteric index less than 10), of which all showed either positive cephalin-cholesterol, delayed bromsulfalein excretion, or reversal of the albumin-globulin ratio. Gall[39] did liver function tests on thirty-three cases serially and found an elevated alkaline phosphatase in all but five cases; twenty-two of twenty-six had positive cephalin-cholesterol tests; fourteen of fifteen had an elevated thymol turbidity; and nine of twenty showed an elevated icteric index (above 8 units). Evans[40] did liver function tests on nineteen consecutive cases without jaundice (thirteen hospitalized and six ambulatory). He found the thymol turbidity elevated in 68 per cent, cephalin-cholesterol positive in 95 per cent, alkaline phosphatase increased in 43 per cent, and in three cases in which electrophoretic determinations were done on the serum he found an increased amount of beta and gamma globulin.

Shay and associates[41] found an elevated thymol turbidity and positive cephalin-cholesterol and serum colloidal gold tests in some cases of infectious mononucleosis. Carter and Maclagan[42] found a positive colloidal gold reaction in 95 per cent and an elevated thymol turbidity in 58 per cent of nineteen patients with infectious mononucleosis, but did not feel that this necessarily indicated any hepatic disturbance.

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Kilham and Steigman[43] were the first observers to demonstrate, by direct observation of the liver cells, that the jaundice in patients with infectious mononucleosis was the result of a hepatitis. In their series of twenty patients there were four clinically jaundiced, whereas others had slight hyperbilirubinemia as demonstrated by laboratory tests. A liver biopsy of one of the jaundiced patients showed a well-marked focal hepatitis. They found maximal changes in the portal tracts with loss of liver cells and a well-developed histiocytic reaction with some early proliferation of bile ducts. The sinusoids showed an excess of Kupffer cells and monocytes. They found no evidence of fibrosis. Van Beek and Haex[44] did liver biopsies on a patient with infectious mononucleosis without jaundice and described the changes as similar to those seen in patients with myeloid leucemia with many monocytoid cells and neutrophiles. A few of the cells showed mitosis. They did a repeat biopsy on the same patient three weeks after recovery and found the liver to be entirely normal. Bang and Wanscher[45] did aspiration biopsy on four patients with infectious mononucleosis with jaundice; however, only two of the four had enlarged livers. All of the sections showed pronounced infiltration of the portal areas with lymphocytes, and a few plasma cells, neutrophiles, and eosinophiles. A few of the liver cells showed mitosis, though no necrosis was evident. There was no evidence of bile duct proliferation or fibrosis. It was their impression that the changes were very similar to those seen in acute epidemic hepatitis with, however, less pronounced parenchymatous changes and more interstitial changes.

Ziegler,[46] Davis and co-workers,[47] Fisher,[48] Allen and Kellner,[49] and Peters and associates[50] reported on the histologic picture of the liver of patients with infectious mononucleosis without jaundice or hepatomegaly who were dying of some other cause. The patients of the first three observers had all died of spontaneous rupture of the spleen. In these cases numerous small foci of infiltrations of mononuclear cells were found. Within these foci there was some degeneration of parenchymal cells. Allen and Kellner's patient had been killed in an airplane accident about a week following recovery from infectious mononucleosis. They, also, found many focal areas of infiltrations of mononuclear cells, which were mostly perilobular in distribution. Some of the liver cells showed mitosis. Peters' two patients died as a result of complicating Guillain-Barré's disease. They describe the liver of one of these patients as showing the picture of a moderate hepatitis.

MATERIALS AND METHODS

The present series consisted of forty consecutive patients with infectious mononucleosis admitted to the Students' Health Service* over a fifteen-month period of time in 1946-1947. This does not include patients with infectious mononucleosis of a mild nature that were cared for on an outpatient basis. Also, no patients were included in this group unless clinical criteria and laboratory tests were unequivocal for infectious mononucleosis. All patients at some time during the illness had an absolute lymphocytosis with atypical or leucocytoid lymphocytes and a heterophile titer of 1:112 or higher. Of course it is recognized that a small percentage of patients never develop a positive heterophile titer.

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Heterophile determinations were done according to the method of Davidsohn.[51] A titre of 1:112 was considered as definitely positive. Determination of the prompt direct one-minute and total bilirubin was made by means of the Ducci and Watson[52] modification of the Malloy and Evelyn[53] method. Values of greater than 0.2 mg. per 100 c.c. of serum for the prompt one-minute direct and values of greater than 0.8 mg. per cent for the total delayed direct and indirect reacting bilirubin were considerd elevated. The thymol turbidity test was performed according to the technique recommended by Maclagan.[54] Values greater than 4 units were considered elevated. The thymol flocculation was done according to the technique of Neefe.[55] Values greater than 1+ were considered abnormal. The cephalin-cholesterol flocculation test was performed according to the method of Hanger,[56] and values greater than 1+ at twenty-four hours were considered abnormal. The bromsulfalein test was performed according to the method described by Gaebler[57] using 5 mg. of the dye per kilogram of body weight. The presence of more than five per cent of the dye remaining after forty-five minutes was considered abnormal. The alkaline phosphatase was done according to the method of Bodansky[58] as modified by Alessandri and Ducci.[59] Values greater than 4.0 Bodansky units were considered elevated. Total cholesterol and cholesterol esters were determined according to the method of Sperry and Schoenheimer.[60] A total cholesterol greater than 220 mg. per 100 c.c. of serum and/or a depression of the esters to less than 60 per cent of the total was considered abnormal. The estimation of the urinary Ehrlich reaction was made according to the methods of Watson and co-workers.[61, 62] Ehrlich units of greater than 1.4 per two to four hour specimen were considered abnormal, and excretion of more than 3.5 mg. of urobilinogen per day was considered abnormal. The urinary coproporphyrin was determined according to the method of Schwartz and associates,[63] and the excretion of more than 100 gamma per day was deemed abnormal.

RESULTS

Of the forty patients with unequivocal evidence of infectious mononucleosis included in the present study, twenty-one (Cases 1 to 21, Table I) showed clear-cut signs of hepatic functional impairment as evidenced by three or more positive liver function tests, exclusive of the test for urinary coproporphyrin. A few of the remaining nineteen patients (Cases 22 to 40, Table I) showed questionable evidence of hepatic dysfunction on the basis of one or two mildly positive tests. The frequency of the abnormality of the various tests in the forty cases is shown in Fig. 1.

Only one of the patients with hepatic functional impairment had definite jaundice, and only four of the twenty-one had an enlarged liver. In three of these four it was definitely tender, and there was tenderness in the liver region in one other patient of this group in whom it was not possible to feel the liver. Forty-eight per cent of the group with hepatic functional impairment had splenomegaly which is about the same (53 per cent) as the group without hepatic dysfunction. All of the patients without laboratory evidence of hepatic dysfunction had a definite pharyngitis, whereas three of the twenty-one patients with marked hepatic dysfunction had no pharyngitis at any time, and three additional patients only developed pharyngitis late in the course of the disease when the hepatitis had nearly subsided. The febrile course did not appear to be greatly different in those with or without hepatic dysfunction. The average maximum temperature elevation in the group with hepatitis was 101.7 degrees F. and in the other group, 101.5 degrees F.

Case 22 is of interest in that the patient returned about one week after discharge from the hospital with a complaint of recurrence of malaise, ano-

TABLE I

CASE	DATE	1'B	TB	TT	TF	CC	BSP	UE	UU	UC	AP	TC	%E	HT	L	EL	TL	P
1	5/14	0.2	0.7	5	0	1+	12		13.3	122				1:224	66			+
2	1/16	0.3	0.8	5		2	20	2.6						1:448	79			+
3	12/16	0.8	2.1	4		2	36	4.2						1:1792	58			+
4	7/24	0.2	0.8	3		2	23	1.7		152				1:224	77	+		+
5	6/6	0.5	1.0	12		3	20		13.1	143	11.8			1:896	77			+
	6/13	0.1	0.6	10		3			2.8			154	64					
6	4/24	0.2	0.7	7	1+	4	9	2.5						1:112	68			+
7	2/28	0.1	0.5	7		4	15	0.4	2.1	111				1:112	71			+
8	2/21	3.9	6.3	2		1		29.0			11.0	162	37	1:224	70	+	+	+
	3/3	1.2	2.3	4	0	2		10.0	34.0	364	15.7	270	55					
	3/10	0.7	1.7	8	Tr.	2	17		3.8	171								
	4/14	0.3	1.0	2	0	0		0.4										
9	5/31	0.4	1.0	3		3			15.0	71	12.3	184	62	1:1792	87			0
	6/12	0.1	0.6	2		1		0.7	1.7									
10	1/30	0.4	1.4	7		2	40	2.5						1:448	76			+
	2/4	0.1	0.6	5		1	16	1.1										
	2/21	0.1	0.8	3		1	5											
11	5/25	0.5	0.8	6	2	4	29		7.1	410				1:448	76			+
12	7/25	0.3	1.0	2	0	1	20	1.0						1:112	80			0
13	7/14	2.2	3.5	8	2	4	35	14.0		133				1:896	84			+
	7/21	0.5	0.9	15	1	4	6	1.2										
	7/30	0.3	0.7	5	1	3												
14	7/31	0.5	1.1	7	0	3	23	2.5		283				1:112	69			+*
	8/8	0.2	0.7	2	0	1	6	0.5										
15	8/19	1.0	1.7	3	2	2	30	8.9						1:112	58		+	+
16	11/12	1.1	2.3	7	Tr.	2			1.0	163	16.1	380	61	1:448	80	+	+	+
	11/18	0.6	1.1	6		0			0.3	112		332	58					
	11/24	0.5	1.0	6	0	0					10.7	314	60					
	12/10	0.1	0.6	6	0	0					6.8	254	63					
17	12/5	0.1	0.4	5	4	4	13	0.4			9.3	124	64	1:1792	71			+*
	12/9	0.2	0.6	7	3	4	6	0.1			7.7	110	67					
	12/15	0.1	0.5	6	3	4	2	0.2			4.2	128	70					

18	12/11	0.4	1.1	6	3	4	12	9.9			6.4	162	52	1:448	67			+
19	5/2	0.4	0.8	5		4		2.7						1:224	74			0
20	1/8	0.1	0.8	7	2	3	10	2.7		123	14.6	162	68	1:448	88			+*
	1/15	0.1	0.7	4	Tr.	2	6	0.4										
21	1/8	0.2	1.0	1		1+		2.8						1:896	62	+	+	0
	1/10	0.3	1.4	2	0	2	19		32.5	739								
	1/15	2.1	3.9	6	0	4	36		6.2	115		162	51					
	1/21	0.5	1.2	5	Tr.	3			3.6	91	23.9	182	60					
22	4/26	0.1	0.9	1	0	0	5		1.4	133				1:1792	76	+	+	+
	5/23	0.1	0.7	9	2+	1	2	0.4										
23	10/8	0.0	0.5	2		0	5	0.2						1:448	63			+
24	10/11	0.1	0.6	2		2	3	0.9						1:896	59			+
25	5/30	0.1	0.4	2		1			0.5	45	3.2			1:1792	68			+
26	4/19	0.1	0.3	4		2	2	0.4						1:448	56			+
27	4/14	0.2	1.2	3	0	1	4	0.4	0.9	249	4.0	204	69	1:448	59			+
28	10/5	0.1	0.9	2		2	4		0.8	113				1:448	81	+		+
29	10/12	0.1	0.6	1		3	7	0.7		73				1:448	60			+
30	12/30	0.1	0.6	4		2	9	1.0						1:896	61			+
31	10/8	0.0	0.5	3		1	4	3.0						1:448	62			+
32	5/28	0.1	0.5	3		1			0.3	46				1:224	68			+
33	5/1	0.1	0.4	4		Tr.		0.5			2.2	156	73	1:224	58			+
34	6/9	0.1	0.4	2		Tr.			3.7	71	4.7			1:1792	64			+
35	10/3	0.1	0.5	7		2	4	0.6	4.4	113				1:896	60			+
36	10/8	0.1	0.4	4		1	4	0.2	0.7	63				1:448	64			+
37	5/29	0.1	0.4	3	Tr.	2	2	0.6	1.8	42				1:448	65			+
38	9/28	0.1	0.4	6		2	6	0.2						1:224	63			+
39	1/23	0.1	0.9	4	0	1	5	1.7						1:896	77			+
40	3/2	0.2	0.6	3	0	1	5		3.8	147				1:1792	58	+		+

1′B, One-minute or prompt reacting serum bilirubin (mg./100 c.c.); TB, delayed direct and indirect reacting serum bilirubin (mg./100 c.c.); TT, thymol turbidity (30 min. reading); TF, thymol flocculation (1+ to 4+ at 18 hr.); CC, cephalin-cholesterol flocculation (1+ to 4+ at 24 hr.); BSP, bromsulfalein retention (45 min. after 5 mg./kg. body weight); UE, urobilinogen in urine (Ehrlich units, 2 to 4 hr.); UU, urobilinogen in urine (mg./day); UC, coproprophyrin in urine (gamma/day); AP, alkaline phosphatase (Bodansky units/100 c.c. serum); TC, total serum cholesterol per 100 c.c.; %E, cholesterol esters (per cent of total cholesterol); HT, heterophile antibody titre; L, highest per cent of lymphocytes in a total white blood cell count above 6,000; EL, enlarged liver; TL, tender liver; P, pharyngitis and/or tonsilitis.

*Developed pharyngitis late in course of disease after subsidence of hepatitis.

rexia, and nausea. Examination showed the liver edge to be palpable at the costal margin and moderately tender. This picture, along with the elevated thymol turbidity and the positive thymol flocculation test, may have indicated a relapse with a hepatitis; however, the patient quickly improved on a regime of restricted activity.

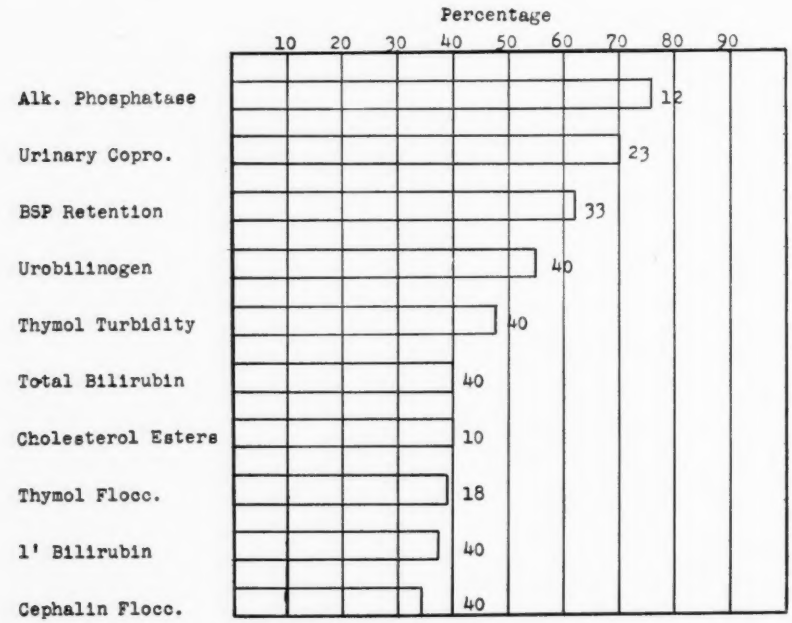

Fig. 1.—Percentage of liver function tests that were positive in a group of forty patients with infectious mononucleosis. The numbers at the ends of the bars indicate the number of patients on whom each test was performed.

In none of the group of forty patients were there any serious complications. In the group with hepatic dysfunction, the liver function tests showed a return to normal in a short period of time. In general the patients with hepatic dysfunction appeared to have a slightly more severe form of disease.

The patients with obvious hepatic involvement were placed on a high protein, high carbohydrate diet, whereas the others were maintained on a general hospital diet. Otherwise the therapy was purely supportive with bed rest when indicated by the temperature and degree of hepatic dysfunction, except for the use of penicillin in a few patients who appeared to have developed an exudative pharyngitis or tonsillitis as a complication.

DISCUSSION

For years there has been a debate in the literature about the possible cause of the jaundice in patients with infectious mononucleosis. Most of the early papers mentioning jaundice as a manifestation of infectious mononucleosis suggested that it was the result of pressure of enlarged lymph nodes on the common bile duct, and was thus an obstructive jaundice.[5, 21, 24, 42] However, as

early as 1928[7] it was suggested that the jaundice was the result of a hepatitis. With the advent of the use of numerous liver function studies for aids in differentiating obstructive and nonobstructive jaundice, it became possible to resolve this question. A number of investigators[36-40] have shown that patients with infectious mononucleosis have the same kind of abnormalities in the liver function tests as patients with epidemic or sporadic infectious hepatitis and homologous serum hepatitis. This work has all pointed to the presence of a hepatocellular and cholangiolar type of liver injury, rather than an extrahepatic obstruction as the cause of the jaundice. It also has been found that the general serum protein disturbances are characteristically the same in infectious mononucleosis as those found in infectious hepatitis, that is, a slight decrease in the albumin and a marked increase in the beta and gamma globulins.[37, 40]

The presence of an intrahepatic cause for the jaundice in these patients received more definitive proof with the histologic demonstration of hepatitis as a result of punch biopsies of the liver of patients with infectious mononucleosis with jaundice[43, 45] and in a patient without jaundice,[44] and also by the demonstration of hepatitis microscopically in patients with infectious mononucleosis without jaundice on autopsy sections of the liver.[46-50]

The present study of forty cases of infectious mononucleosis also demonstrates clearly the presence of both hepatocellular and cholangiolar liver damage as the basis for the functional impairment of the liver and in one case (Case 8) for clinical jaundice. Case 16 was of interest in that the patient exhibited predominately a cholangiolar type of hepatitis, namely increased prompt reacting (one minute) bilirubin, alkaline phosphatase, total cholesterol, and the presence of an intense pruritis.

Thus it would appear that even though jaundice is not especially common in infectious mononucleosis, hepatitis without jaundice is extremely common. The criticism might be ventured, however, that this group of patients is not particularly representative of patients with infectious mononucleosis, and that the degree of liver involvement is out of proportion to that seen in the usual hospitalized patient with infectious mononucleosis. Yet the percentage of patients in this series with jaundice (2.5 per cent) is no higher than reported by other investigators: 10,[4] 12,[8] 7,[9] 5,[13] 4,[23] 2,[26] 3,[27] 5,[28] 1.4,[29] 7.7,[30] 3.3,[31] and 7.5 per cent.[36] The percentage of patients with hepatomegaly might also give some clue as to the probable incidence of hepatic involvement in other reported series. It was 15 per cent in the present group of forty cases. Others have variously reported the incidence at 27,[8] 33,[10] 13,[13] 16,[31] 26,[33] and 17 per cent.[36]

It is fair to assume that one would anticipate more patients with hepatitis without jaundice than patients with jaundice. It has been demonstrated in the development of experimental hepatitis in human volunteers that the number of patients that develop hepatitis without jaundice exceeds the number that develop jaundice.[64]

The criticism might be anticipated that one could get the degree of hepatic involvement seen in these cases in simple upper respiratory infections or cer-

tain relatively benign viral infections. However, it has been shown by others[49, 52] that such is not the case, nor have I observed this degree of functional impairment of the liver in the pharyngitides and upper respiratory infections. Also, four of these patients with hepatitis had no pharyngitis at any time and very little fever, and three others did not develop the pharyngitis until after the hepatitis had nearly subsided. For hyperthermia alone to cause a marked degree of liver functional impairment it is necessary for the patient to be exposed to a severe and prolonged hyperthermia.[65, 66]

A patient with acute infectious mononucleosis and hepatitis and a patient with acute epidemic or sporadic infectious hepatitis often pose a difficult problem in differential diagnosis. Clinically the one symptom that is most valuable in differentiating the two conditions is sore throat. Most all patients with infectious mononucleosis will complain of a moderate to severe sore throat during some period of the illness, whereas this is not a very outstanding sign in infectious hepatitis. As far as physical signs are concerned this is also a valuable guide. There were only four patients out of the entire group of forty that did not have a definite pharyngitis at some stage of illness. All four of these were in the group with definite hepatic dysfunction. This pharyngitis is usually of the nonexudative, nonspecific type, but in some cases is of the exudative type and beta hemolytic streptococci can be isolated from the throat. Lymph node enlargement is distinctly more common and more marked in infectious mononucleosis than in infectious hepatitis; however, the difference is not too striking. Various investigators have listed splenomegaly in infectious hepatitis as 20,[64] 13,[67] and 21 per cent.[68] In our cases of infectious mononucleosis we have found splenomegaly in 48 per cent, which is comparable to what other observers have found: 50,[4] 47,[28] and 34 per cent.[31]

Conversely, the liver is probably much more often enlarged in infectious hepatitis than in infectious mononucleosis. However, Zimmerman and co-workers[68] found the liver to be enlarged in only 69 per cent of a series of patients with infectious hepatitis in which 90 per cent of the patients were jaundiced. There are few papers on infectious hepatitis without jaundice, so it is difficult to make a comparison with patients with infectious mononucleosis without jaundice. Barker and associates[64] and Finks and Blumberg[69] state that in infectious hepatitis without jaundice the liver is usually enlarged. Fifteen per cent or six of the forty patients in this group showed hepatomegaly, and four of these were in the group with hepatitis. Others have reported similar figures; 16 per cent,[31] and 17 per cent.[36]

Thus it may be seen that there is considerable overlapping in the clinical picture in these two conditions, and in the final analysis one must rely on laboratory tests to differentiate the two. Patients with both infectious hepatitis and infectious mononucleosis show the atypical or leucocytoid lymphocytes of Downey,[70] Types I, II, and III—mostly Type II. However, patients with infectious mononucleosis have a much greater number and at some stage in the disease practically always have an absolute lymphocytosis with large numbers of leucocytoid lymphocytes, whereas patients with infectious hepatitis only de-

velop a relative lymphocytosis. It has been found that in experimentally induced infectious hepatitis in human beings there is only a relative lymphocytosis with the greatest number of atypical lymphocytes at the fourth to fifth day after the onset of fever.[71] In infectious mononucleosis the lymphocytosis usually is not transient but increases in degree as the disease progresses and usually remains absolute after the symptoms have subsided.

The heterophile antibody titer is equally as helpful as the lymphocytic reaction in differentiating the two conditions. It is practically always positive in infectious mononucleosis if enough determinations are obtained, whereas we have never found it to be positive in infectious hepatitis. Others also have been unable to find an elevated heterophile titer in infectious hepatitis.[37, 38, 40, 49, 64] Eaton and associates[72] noted a moderate increase in titer of antibodies to sheep red blood cells in some patients with infectious and homologous serum jaundice. These antibodies, however, were of true Forssman type, adsorbed by guinea pig kidney, thus unlike those of infectious mononucleosis.

From the present study it is apparent that liver function tests are of little value in differentiating infectious hepatitis from infectious mononucleosis with hepatic functional impairment. The liver profiles of the patients with infectious mononucleosis are for the most part characteristic of those seen in infectious hepatitis. Fig. 1 shows graphically the tests that were most frequently positive in the patients with infectious mononucleosis, and in general these are the same tests that one finds positive in the greatest percentage in patients with infectious hepatitis.

The urinary coproporphyrin excretion is increased in both diseases, but is of no value in differentiating the two because the increase in both is of the Type I isomer. This is considered in more detail elsewhere.[73, 74]

In this series of patients with infectious mononucleosis, as in patients with infectious hepatitis,[75, 76] the increase of serum bilirubin is due to an increase of the prompt reacting type more than the delayed or indirect. Evans[49] found the cephalin-cholesterol test a more sensitive indicator of hepatic dysfunction than the thymol turbidity test in infectious mononucleosis. In this series the reverse has been found to be true. Also, the thymol test remains positive longer than any of the other tests; this also has been found to occur in cases of infectious hepatitis.[76, 77] In the present series there is likewise shown to be a better agreement between the thymol flocculation and cephalin-cholesterol flocculation than between the thymol turbidity and thymol flocculation. This is contrary to what others have found in infectious hepatitis.[76] It also has been reported as unusual for the thymol turbidity test to be positive in the absence of a positive thymol flocculation test in infectious hepatitis.[76] However that has not been the case in the present group of cases of infectious mononucleosis.

SUMMARY

A series of forty cases of typical infectious mononucleosis has been studied in which twenty-one (55 per cent) of the patients showed moderate to severe hepatic functional involvement or hepatitis as evidenced by a battery of liver

function tests. A number of additional patients in the group exhibited milder hepatic involvement on the basis of one or two positive tests.

It has not been found feasible to differentiate the type of liver involvement in these patients with infectious mononucleosis and hepatic dysfunction from that in cases of acute epidemic or sporadic hepatitis. One must rely on the clinical picture, especially the presence of pharyngitis, the type of lymphocytic reaction, and the titer of the heterophile antibodies. The character of the liver function disturbances in these patients points to the presence of both hepatocellular and cholangiolar liver injury. This evidence together with the histologic findings reported in the literature tends to refute the theory that jaundice in infectious mononucleosis is due to an extrahepatic obstruction of the common bile duct by enlarged lymph nodes.

The literature on the subject of hepatic involvement in infectious mononucleosis is reviewed.

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FOLIC ACID METABOLISM STUDIES

III. Intravenous Administration of Pteroylglutamic Acid and Pteroyltriglutamic Acid

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With the Technical Assistance of Frances Panzer and Patricia Sparks

RECENT studies[1] revealed that conjugases present in blood were capable of releasing folic acid from combined forms of the vitamin in blood or from pteroylheptaglutamic acid. This was evidenced by an increase in the folic acid content obtained when blood from various animals, including man, was incubated at pH 7.0 as compared with the values obtained before incubation.

Considerable interest has developed in determining the comparative effectiveness of folic acid and related compounds in the treatment of macrocytic anemias and related diseases.[2] Excretion studies conducted with normal subjects have shown that negligible amounts (less than 10 μg per day) are excreted in the urine when average diets are ingested. The urinary excretion of the vitamin is markedly increased, however, when oral or intravenous supplements of pteroylglutamic acid or its conjugates are administered.[3-7] The major portion of the dose is excreted in the first twenty-four hours.

The effect of the amount of folic acid ingested on the folic acid content of the tissues, including blood, has been investigated with various animal species.[8-13] The apparent free folic acid in the blood of the turkey, for example, is markedly reduced when a diet low in the vitamin is given.[11, 13]

In the present work, the effect of administering folic acid intravenously, either as pteroylglutamic acid or as pteroyltriglutamic acid, to human subjects on the blood levels of the vitamin was determined at different time intervals after injection. These studies have afforded information on the comparative effectiveness of the two forms of the vitamin in maintaining the blood level and evidence for the rapid cleavage of pteroyltriglutamic acid to compounds active for the test organism, *Streptococcus faecalis* R.

EXPERIMENTAL

Normal adult human beings consuming average diets were used in this study. Six subjects were used in each of two series of tests. Three subjects in each series received pteroylglutamic acid and three received pteroyltriglutamic acid. Two of the three subjects in each group also were used in the second series conducted two weeks later, and the test compound administered was the reverse of that given in the first series. Blood samples

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We are indebted to Dr. T. H. Jukes, Lederle Laboratories, American Cyanamid Company, Pearl River, N. Y., for suggesting this problem and for generous supplies of pteroylglutamic acid and pteroyltriglutamic acid. The cooperation of R. E. Simpson, George Barron, Carl Widmer, A. K. Sparks, Juanita Pou, Dr. R. T. Holman, and the staff members of the College Hospital in the course of these studies is greatly appreciated.

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were taken by vein before injection of the test compound and two, four, eight, and twenty-four hours later. Pteroylglutamic acid at a level of 12 mg. or the equivalent on a molar basis of pteroyltriglutamic acid was injected intravenously.

Appropriate dilutions of the oxalated whole blood from each subject at each time interval were made. The blood samples were autoclaved to inactivate the blood conjugases[1] and prepared for assay in the usual manner. The folic acid content was determined after each treatment with *S. faecalis* R as the test organism.[14] In one of the series, comparative results were obtained when *Lactobacillus casei*[14] was used as the test organism. The amount of folic acid per milliliter of blood is shown graphically in Fig. 1 for the *S. faecalis* R assays and in Fig. 2 for the *L. casei* assays. The individual values for each subject obtained at each time interval are also indicated.

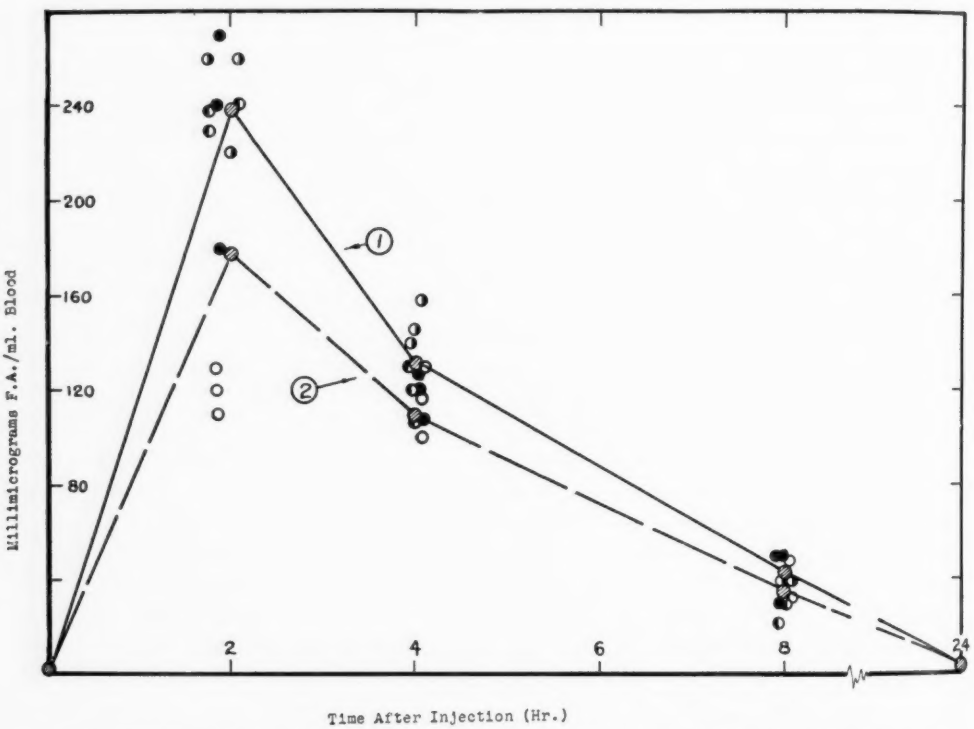

Fig. 1.—Effect of administering pteroylglutamic acid or pteroyltriglutamic acid on the level of folic acid in the blood measured with *S. faecalis* R as the test organism. *1*, Pteroylglutamic acid: ● ●, first series; ◑ ◑, second series. *2*, Pteroyltriglutamic acid: ◐ ◐, first series; ○ ○, second series. The crosshatched circles represent the averages for the six tests conducted at each time interval.

RESULTS AND DISCUSSION

The folic acid of the blood as determined includes only those compounds that have activity for the test organisms without treatment of the sample. Pteroylglutamic acid is equally active for both microorganisms. Pteroyltriglutamic acid is essentially inactive for *S. faecalis* R but is quite active for *L. casei*.[2] Therefore in order for pteroyltriglutamic acid to elicit a response with *S. faecalis* R, this vitamin derivative must be cleaved to pteroylglutamic acid or other derivatives which can be utilized by *S. faecalis* R. For *L. casei*, how-

ever, both test compounds are active and cleavage of pteroyltriglutamic acid is not necessary for microbiologic activity. On the other hand, if pteroic acid is formed it would be essentially inactive for *L. casei* but active for *S. faecalis* R.

It will be noted that the level of the free vitamin in the blood as determined with both test organisms is very low before injection and that it returned to this low level twenty-four hours after injection. For many of the subjects the free folic acid level was too low to measure (<0.6 millimicrograms per millimeter). A prompt and rapid rise in the blood levels as determined with

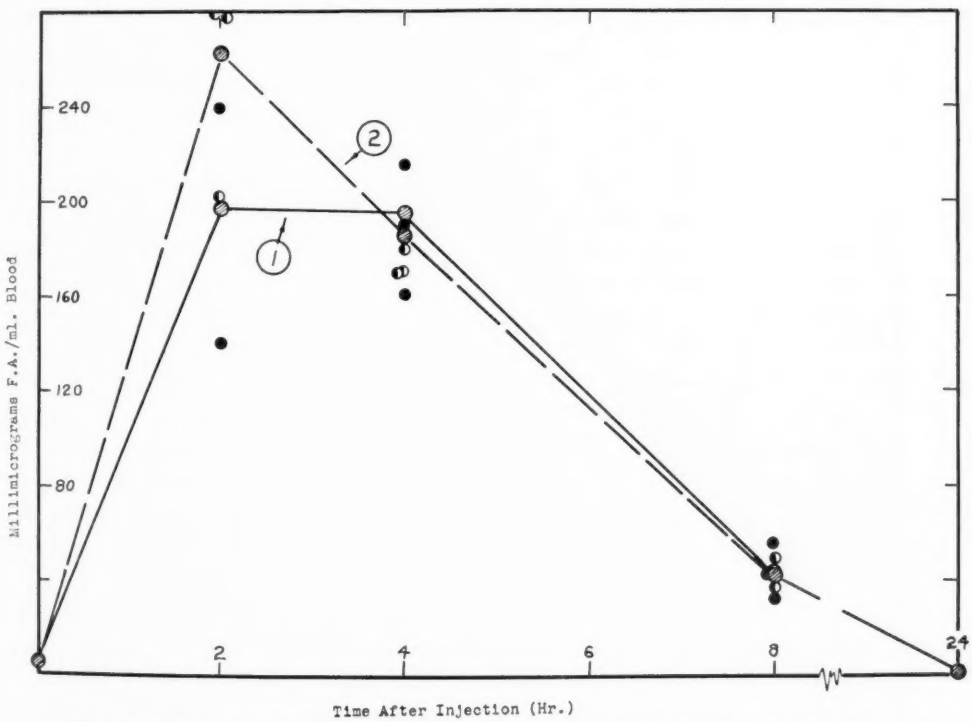

Fig. 2.—Effect of administering pteroylglutamic acid or pteroyltriglutamic acid on the level of folic acid in the blood measured with *L. casei* as the test organism. *1*, Pteroylglutamic acid: ● ●, first series. *2*, Pteroyltriglutamic acid: ◐ ◐, first series. The crosshatched circles represent the averages for the three tests conducted at each time interval.

S. faecalis R occurred after injection and was at a maximum two hours after injection, with a decrease observed in the blood levels four and eight hours after injection (Fig. 1). The data obtained with *L. casei* (Fig. 2) indicate a high level of the vitamin at two and four hours after injection, with a rapid fall occurring at eight hours after injection.*

The values obtained for the different subjects at each time interval are in rather good agreement when *S. faecalis* R was used as the test organism with the exception of those obtained two hours after pteroyltriglutamic acid

*The data do not permit a definite statement as to the time after injection when the maximum blood levels were reached, but are discussed as comparisons at the intervals when analyses were made. The data do show that the maximum blood levels were reached within four hours after the injection of both vitamin derivatives so measured with *S. faecalis* R.

was injected. The values obtained in the second series appear less reliable, since they were calculated from data obtained from the low portion of the standard curve and since they do not indicate clearly a decrease when compared with the data obtained four hours after injection. At any rate, both compounds were effective in raising the blood level of folic acid, and pteroyltriglutamic acid was utilized essentially as well as pteroylglutamic acid. The over-all response was essentially the same for each subject, and the same was true whether the subject received pteroylglutamic acid in the first or second series of tests.

It is also apparent that conjugases presumably of the blood and possibly from other tissues were highly effective in splitting pteroyltriglutamic acid into products that were highly active for *S. faecalis* R. The ability of conjugases from human blood to release active components from pteroylheptaglutamate has been described in a previous paper.[1]

The values obtained with *L. casei* before injection and twenty-four hours thereafter were in essential agreement with those obtained with *S. faecalis* R. The values obtained four hours after injection were usually higher with *L. casei* as the test organism, particularly when the triglutamate was administered, suggesting that some of the injected triglutamate was not cleaved for as long as four to eight hours after injection. The over-all curves for the values obtained with the two organisms when either pteroylglutamic acid or pteroyltriglutamic acid was administered correlate very well however (Figs. 1 and 2).

It is of interest that an increase in the urinary excretion of folic acid following the ingestion of pteroylglutamic acid or pteroyltriglutamic acid also occurred only for the first twenty-four hours after administration.[7] These techniques should be valuable for further studies not only on the metabolism of pteroylglutamic acid and related compounds by normal human beings, but also on the metabolism of these important nutrients by patients with diseases that respond to treatment with folic acid and other hematopoetic substances. Since the free folic acid level is very low in the blood of normal human beings receiving normal diets, this measurement alone would not be useful in assessing the nutritional state with respect to folic acid. Additional studies comparing the values obtained with different test organisms for determining folic acid will afford data on the nature of the compounds present in various tissues and body fluids.

SUMMARY

The level of folic acid in the blood of human subjects was determined with *S. faecalis* R and *L. casei* as the test organisms two, four, eight and twenty-four hours after the intravenous administration of 12 mg. of pteroylglutamic acid or the equivalent of pteroyltriglutamic acid. A prompt, rapid, and essentially equal rise in the blood levels occurred when these compounds were given. The level was maximum two hours after injection when the first analyses were made, was still elevated eight hours after injection, but was at a normal level twenty-four hours after injection.

The values obtained with *L. casei* four hours after injection of the vitamin were somewhat higher than those obtained with *S. faecalis* R when pteroyltriglutamic acid was administered, indicating that some of the triglutamate was present in the blood for at least four to eight hours after injection. These data also show, however, that conjugases of the tissues were capable of a rapid and effective release of derivatives active for *S. faecalis* R from pteroyltriglutamic acid.

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STUDIES IN SERUM PROTEINS

IV. Clinical Studies Employing Rapid Chemical Fractionation Procedures, With Particular Reference to the Frequency and Significance of Hypoalbuminemia

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DETERMINATIONS of "albumin" by salt fractionation procedures have shown this fraction to be decreased in a number of clinical conditions.[1] However, the "albumin" values obtained by such methods are now known to include at least two of the fractions revealed by electrophoretic analysis, namely albumin and alpha globulin. For this reason, and because albumin and alpha globulin have been shown to bear an inverse relationship to each other,[11] a review of the frequency and magnitude of hypoalbuminemia in clinical conditions has appeared desirable.

Electrophoretic investigations have shown a significant decrease in albumin concentration in certain diseases,[1] but because of the limited number of samples which can be handled by an electrophoretic laboratory, the available data do not yet give a true picture of the frequency with which the various conditions responsible for hypoalbuminemia actually occur in clinical practice. Chemical fractionation methods have recently been shown to give results comparable to those obtained by electrophoresis[2, 3] and also to permit analysis of large numbers of samples on a routine basis.[4] The results to be discussed were obtained by the application of our chemical fractionation method to 500 routine sera.

METHODS

The general principles of the fractionation procedure used are as follows:

1. Total protein is estimated with the aid of Weichselbaum's biuret reagent.

2. A filtrate containing albumin plus alpha globulin is obtained by treating serum with sodium sulfate at a final concentration of 20.24 per cent. Protein in the filtrate is estimated by the biuret reaction.

3. A filtrate containing albumin alone is obtained by treating serum with sodium sulfite at a final concentration of 26.88 per cent. Protein in the filtrate is estimated by the biuret reaction.

4. The alpha globulin value is obtained by subtracting the albumin concentration from that of albumin plus alpha globulin.

5. Beta globulin plus gamma globulin is calculated by subtracting the albumin plus alpha globulin concentration from the total protein concentration.

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6. Gamma globulin is precipitated from serum by ammonium sulfate at a final concentration of 1.39M, at pH 6.0 and controlled ionic strength. The precipitate is dissolved in dilute biuret reagent for the estimation.

7. Beta globulin is calculated by subtracting the concentration of gamma globulin from that of beta plus gamma globulin.

The beta plus gamma globulin component was not further fractionated in this group of patients, except in the supplementary group of nephrotic patients presented in Tables IV and V. Complete globulin fractionations are now being performed on a second group of patients and will be reported later.

Detailed working instructions for the rapid estimation of total protein, albumin, total globulin, alpha globulin, beta globulin, and gamma globulin in 1.0 ml. of serum will be published shortly.[4] The time required to carry through the entire procedure is only slightly more than twice that required for a determination of total protein and albumin by the Howe method.

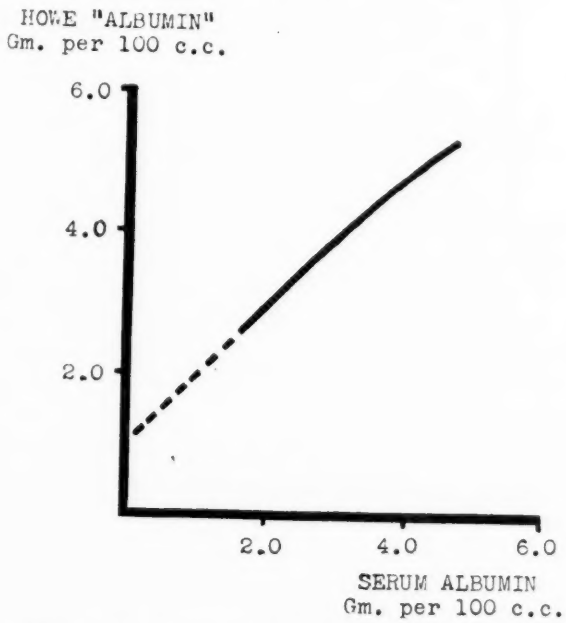

Fig. 1.—Average relation between Howe "albumin" and serum albumin values in 500 unselected sera.

RESULTS

Comparison of Howe "Albumin" Values With Albumin Values.—It is now generally recognized that the "albumin" values obtained by the Howe sodium sulfate fractionation are always somewhat larger than the albumin values obtained by electrophoresis or by chemical fractionation techniques which have been adjusted to electrophoretic standards.[2-9*] The difference between the Howe

*For the purposes of clarity, we have called the values obtained by the Howe procedure "albumin," and those obtained by electrophoretic procedures or by chemical methods adjusted to electrophoretic standards, albumin. This does not imply that the albumin fraction isolated by electrophoresis is necessarily a unitary fraction except in its behavior in an electrical field and under suitable precipitation conditions. There is, in fact, reason to believe that it may consist of at least two major subfractions and several other minor components.

"albumin" values and albumin values has been shown to be approximately equal to the alpha globulin concentration[2, 7] and to vary with the alpha globulin concentration.[2] In this series of 500 hospital patients, the average serum alpha globulin was 0.84 Gm. per cent, with two-thirds of the values within the range of 0.6 to 1.1 Gm. per cent. Most of the Howe "albumin" values were therefore from 0.6 to 1.1 Gm. per cent greater than the albumin values determined simultaneously. Because this variation in alpha globulin values is not large in comparison with the size of the albumin fraction, the correlation between Howe "albumin" values and albumin values (Fig. 1) is approximately linear.

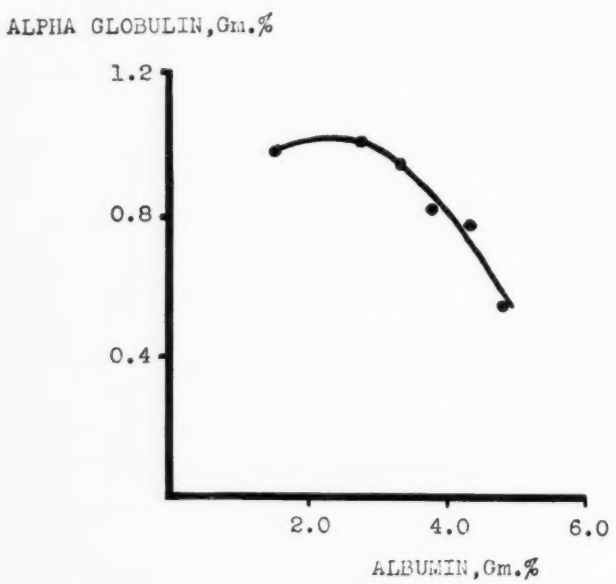

Fig. 2.—Average relation between albumin and alpha globulin in 500 unselected sera. (Each point represents the average of approximately eighty determinations.)

Attempts to apply these data correlating Howe "albumin" values and albumin values to the derivation of a constant correction factor by which albumin may be calculated from Howe "albumin" values meet with certain definite objections, particularly when the accuracy of individual determinations is considered. Although the majority of alpha globulin values in our series fell in the middle range of 0.6 to 1.1 Gm. per cent, eight patients had concentrations of 0.2 Gm. per cent or less, and sixteen patients had concentrations of 1.5 Gm. per cent or greater. Moreover, study of large numbers of samples has shown a definite tendency for alpha globulin concentrations to increase both absolutely and relatively as serum albumin concentrations decrease,[11, 12, 13] although the scatter of the data both in our series and in that of Chow[11] is very large.

Fig. 2 shows the average correlation between serum albumin and alpha globulin values which obtains in our patients. At an average serum albumin of 4.81 Gm. per cent, the alpha globulin concentration averaged 0.54 Gm. per cent, and this value increased to an average of 1.00 Gm. per cent at a serum

albumin concentration of 2.76 Gm. per cent. Further decreases in albumin concentration did not lead to additional increases in the absolute values of alpha globulin, even though its relative concentration continued to increase. Because those circumstances which lead to a decrease in albumin values also usually lead to increased alpha globulin values, the net change in the Howe "albumin," which includes both albumin and alpha globulin, will tend to be less than the change in the albumin value.

A more serious defect of the Howe "albumin" values may be deduced from Fig. 1, where the line which represents the relationship between Howe "albumin" values and true albumin values has been extrapolated to the base line. This projection, shown as an interrupted line, indicates that, at an average true albumin concentration of 0.0 Gm. per cent, the Howe "albumin" value will still be above 1.0 Gm. per cent. It suggests that examination of a large series of samples should show the lowest Howe "albumin" values to be about 1.0 Gm. per cent. This suggestion appears to have been borne out in our series of 500 determinations. Two patients showed Howe "albumin" values between 0.9 and 1.0 Gm. per cent, but all others had values of 1.30 Gm. per cent or greater. This finding, when considered with the data on the frequency of hypoalbuminemia discussed below, indicates that Howe "albumin" values conceal both the frequency and the magnitude of hypoalbuminemia.

Findings in Hypoalbuminemia.—In this series hypoalbuminemia has been defined as a serum albumin concentration of 2.0 Gm. per cent or less. Such values occurred in fifty samples from thirty-four patients, an incidence of 9.8 per cent. In twenty samples from ten patients (incidence 3.8 per cent), the albumin concentration was 1.0 Gm. per cent or less. Howe "albumin" values were below 2.0 Gm. per cent in only ten patients, and 1.0 Gm. per cent or less in only two patients.

Electrophoretic studies have shown that marked hypoalbuminemia may occur in severe malnutrition, in the nephrotic syndrome, in chronic tuberculosis, with severe hepatic damage, during relapsing *Plasmodium vivax* malaria,[1, 14-20] and in the syndrome of idiopathic familial dysproteinemia.[16, 21, 22*] Examples of each of these disorders, except for the last two, were found during this study, and a few additional diagnoses were noted.

Table I lists the diagnoses made in our cases of hypoalbuminemia and the frequency of each. Quantitatively, the largest group was that of patients with the nephrotic syndrome. Two patients in this group had severe renal amyloidosis associated with long-standing tuberculosis, but the remainder of the patients

**Dysproteinemia* is a term which has been used to designate an abnormal distribution of the normal plasma protein components, and is contrasted with *paraproteinemia* in which abnormal constituents are believed to appear in the plasma, as in myelomatosis.[16] The syndrome of familial idiopathic dysproteinemia has been described by Homburger, Young, Petermann, and Reifenstein[22] as consisting of the familial occurrence of grossly abnormal plasma protein patterns, not necessarily with manifest hypoproteinemia. Clinical signs included edema and peripheral circulatory abnormalities in affected women.

Armstrong[21] has referred to a group of similar cases as idiopathic hypoproteinemia and distinguishes two types: one in which the primary disturbance appears to be abnormal plasma protein formation; the other in which increased capillary permeability appears primary.

Wuhrmann and co-workers[16] also distinguish two types: one in which there is hypoproteinemia, but with a normal electrophoretic distribution of components; the other in which the electrophoretic distribution resembles that of the nephrotic syndrome (nephrosis sine albuminuria). They have found that both types may be intermittent, edema appearing and diminishing with regular cyclic rises and falls in serum protein values.

were believed to suffer from chronic glomerulonephritis. No attempt was made to differentiate chronic from subacute glomerulonephritis. Three patients were diagnosed clinically as having hepatic cirrhosis. A third group of four patients was classified as having severe gastrointestinal involvement. This noncommittal designation was chosen because it was difficult to assess the relative importance of deficiencies in food intake or absorption and of functional hepatic impairment in the genesis of the hypoalbuminemia.

TABLE I. CLINICAL DIAGNOSES IN THIRTY-FOUR PATIENTS PRESENTING SERUM ALBUMIN CONCENTRATIONS OF 2.0 GM. PER CENT OR LESS

Nephrotic syndromes	
Nephrotic stage, chronic glomerulonephritis	18
Renal amyloidosis, secondary to tuberculosis	2
Severe hepatic dysfunction	
Hepatic cirrhosis	3
Severe gastrointestinal involvement	
Carcinoma of gastrointestinal tract	2
Intestinal obstruction	1
Following gastric resection	1
Disorders with reticulo-endothelial involvement	
Multiple myeloma	3
Hodgkins' disease	1
Dermatomyositis	1
Rheumatic fever	1
Idiopathic lymphatic atrophy with lymphopenia and hypogammaglobulinemia	1

A final group* of patients were of interest because they presented a variety of conditions in which the reticulo-endothelial system was markedly involved and in which quantitative and qualitative alterations in serum globulins are ordinarily much more pronounced than hypoalbuminemia. This has been true in our study as well; most of our patients with multiple myeloma or rheumatic fever did not show a severe degree of hypoalbuminemia. Although at present the liver is believed to be the sole source of serum albumin,[23, 24] it is not yet established that the hypoalbuminemia seen in nephrotic patients with mild proteinuria, or in patients with reticulo-endothelial involvement, is to be taken as implying structural liver damage.

Table II lists the average values for the various serum protein fractions found in each of the groups mentioned and for the entire group of patients.

Hypoalbuminemia is most striking in the nephrotic syndrome. (Table III.) The average serum albumin concentration of the nephrotic patients was only 0.93 Gm. per cent, while average serum albumin values exceeded 1.60 Gm. per cent in all other groups. *Serum albumin concentrations of 1.0 Gm. per cent or less were seen only in the nephrotic syndrome.* Although these concentrations appear small, our data show good agreement with the results of the few reported electrophoretic studies, which are summarized in Table IV.

The absolute values of the alpha globulin and the beta plus gamma globulin fractions of the nephrotic sera was only slightly larger than those of the average hospital patient. Possibly because of the tendency for gamma globulin to be lost in the urine with albumin in nephrosis, gamma globulin values in these

*One patient who presented hypogammaglobulinemia, lymphopenia, lymphatic atrophy, and bronchiectasis will not be discussed in detail in this report. Complete data will be presented in a forthcoming case report.

TABLE II. MEAN VALUES FOR SERUM PROTEIN FRACTIONS IN PATIENTS WITH HYPOALBUMINEMIA

DIAGNOSIS	NUMBER	TOTAL PROTEIN (GM. %)	ALBUMIN (GM. %)	TOTAL GLOBULIN (GM. %)	ALPHA GLOBULIN (GM. %)	BETA PLUS GAMMA GLOBULIN (GM. %)	A/G RATIO
Nephrotic syndrome*	20	4.55	0.93	3.62	1.15	2.53	0.25
Gastrointestinal involvement	4	5.55	1.74	3.81	1.06	2.75	0.46
Reticulo-endothelial system involvement							
Multiple myeloma	3	7.32	1.87	5.45	----	----	0.34
Other causes*	3	6.60	1.63	4.97	0.80	4.10	0.33
Hepatic dysfunction	3	6.58	1.85	4.73	0.82	3.91	0.39
Hypogammaglobulinemia with lymphatic atrophy	1	3.50	2.00	1.50	0.90	0.60	1.33
Hospital patients (unselected)	400	6.40	3.29	3.11	0.84	2.27	1.06
M.R.H.§ pooled normal serum	†	7.01	3.77	3.24	1.10	2.14	1.16
Normal adults	‡	6.58	3.42	3.16	1.06	2.10	1.08

*Total globulin values differ slightly from the sum of the globulin components because globulin fractionation was not performed in one or more patients.

†Average data from several determinations carried out on each of four samples of pooled normal human serum. Each pool contained sera from between 30 and 150 individuals. Chemical fractionation on two of the pools was checked by electrophoretic runs.

‡Data from the Harvard Plasma Fractionation Program[29, Table IX] recalculated for the omission of fibrinogen.

§Michael Reese Hospital Serum Center.

patients may be markedly diminished, and average gamma globulin values as low as 0.24 Gm. per cent have been recorded in electrophoretic analyses.[16, 20, 25-28] Gamma globulin levels in amyloidosis, however, tend to approach normal values,[16, 26, 27] and a tendency for gamma globulin values to rise during exacerbations of the nephrotic syndrome has been reported.[20, 27] The relatively normal values of serum gamma globulin in amyloid nephrosis may be associated with the presence of large amounts of gamma globulin in the urine.[27]

Chemical fractionation values on twenty samples from sixteen nephrotic children are presented in the lower portion of Table III. The average gamma globulin concentration is 0.30 Gm. per cent, a value in close agreement with the value of 0.33 Gm. per cent obtained by averaging the reported electrophoretic data. (Table IV.) There is a striking correlation of albumin and gamma globulin values. Ten children who had serum albumin concentrations below 1.0 Gm. per cent had an average gamma globulin concentration of 0.11 Gm. per cent, while six children with serum albumin concentrations above 1.0 Gm. per cent had an average gamma globulin concentration of 0.62 Gm. per cent. This result does not depend upon technical factors in the salting out method since we have shown that serum albumin concentrations do not affect the completeness of gamma globulin recovery by the method employed.[4] It would appear, therefore, that whether or not proteinuria is considered to be the sole cause of hypoalbuminemia in nephrosis, the same factors which are responsible for severe hypoalbuminemia are also responsible for severe hypogammaglobulinemia.

As a consequence of the decreased gamma globulin concentrations, the beta plus gamma globulin fraction, which is roughly equivalent to the Howe "globulin,"[2] comes to consist almost entirely of beta globulin in the average nephrotic

patient (Tables III and IV). The serum beta globulin concentrations show an inverse relationship to the serum albumin concentration. The ten children in Table III with serum albumin values below 1.0 Gm. per cent had an average

TABLE III. SERUM PROTEIN FRACTIONATION IN PATIENTS WITH THE NEPHROTIC SYNDROME
(The samples in the lower portion of the table, for which complete fractionation data are given, were studied after conclusion of the series of 500 determinations with which the paper is chiefly concerned)

PATIENT	NUMBER OF SAMPLES	TOTAL PROTEIN (GM. %)	ALBUMIN (GM. %)	TOTAL GLOBULIN (GM. %)	ALPHA GLOBULIN (GM. %)	BETA PLUS GAMMA GLOBULIN (GM. %)	BETA GLOBULIN (GM. %)	GAMMA GLOBULIN (GM. %)	A/G RATIO
Etiology: Chronic Glomerulonephritis									
A. N. S.*	2	4.85	0.15	4.70	0.78	3.92	---	---	0.03
S. P. I.*	2	4.70	0.15	3.95	1.55	2.40	---	---	0.03
B. R. O.*	1	4.80	0.30	4.50	---	---	---	---	0.07
F. E. L.*	5	3.62	0.38	3.24	1.10	2.14	---	---	0.12
K. A. P.*	1	3.80	0.40	3.40	0.90	2.50	---	---	0.12
F. O. R.*	2	3.60	0.40	3.20	0.50	2.70	---	---	0.13
B. R. Z.	1	4.05	0.80	3.25	1.60	1.65	---	---	0.25
H. O. W.*	1	5.20	0.80	4.40	1.00	3.40	---	---	0.18
N. I. S.*	4	4.78	0.93	3.85	0.83	3.02	---	---	0.25
G. U. E.*	1	5.00	1.00	4.00	---	---	---	---	0.25
G. R. A.*	1	6.30	1.10	5.20	1.40	3.80	---	---	0.21
W. I. L.*	1	4.40	1.10	3.30	---	---	---	---	0.33
D. A. H.	1	4.10	1.10	3.00	1.00	2.00	---	---	0.37
S. E. D.*	1	3.40	1.10	2.30	0.70	1.60	---	---	0.48
P. A. N.	2	3.55	1.35	2.20	0.55	1.65	---	---	0.70
W. Y. Z.	1	5.50	1.40	4.10	1.30	2.80	---	---	0.34
B. O. R.*	1	4.30	1.40	2.90	1.20	1.70	---	---	0.48
E. K. T.	1	4.00	1.80	2.20	0.90	1.30	---	---	0.82
Etiology: Amyloidosis During Chronic Tuberculosis									
L. U. N.	1	5.40	1.00	4.40	1.20	3.20	---	---	0.23
W. A. S	1	6.40	2.00	4.40	1.20	3.20	---	---	0.46
Average of 20 patients	*31*	*4.55*	*0.93*	*3.62*	*1.15*	*2.53*	---	---	*0.25*
Etiology: Chronic Glomerulonephritis (Complete Protein Fractionations)									
S. P. I.*	2	3.40	0.10	3.30	1.35	1.95	1.67	0.28	0.03
G. R. A.*	1	3.20	0.20	3.00	1.10	1.90	1.80	0.10	0.07
C. C. C.*	1	3.80	0.20	3.60	1.05	2.55	2.40	0.15	0.05
S. E. D.*	1	4.40	0.20	4.20	1.70	2.50	2.35	0.15	0.05
Y. O. U.*	1	4.30	0.30	4.00	1.70	2.30	2.21	0.09	0.08
A. N. S.*	1	4.40	0.30	4.10	0.60	3.50	3.35	0.15	0.07
C. C. B.*	1	3.30	0.40	2.90	0.85	2.05	1.90	0.15	0.14
F. O. R.*	1	3.40	0.40	3.00	1.00	2.00	1.90	0.10	0.13
S. I. L*	2	4.60	0.70	3.90	1.35	2.55	2.17	0.38	0.18
H I. G.*	1	3.70	0.90	2.80	1.20	1.60	1.10	0.50	0.32
G. U. E.*	2	5.15	1.00	4.15	1.35	2.80	1.40	1.40	0.24
C. C. A.*	1	3.80	1.10	2.70	0.90	1.80	1.45	0.35	0.41
C. C. E.*	1	3.70	1.30	2.40	0.90	1.50	0.80	0.70	0.54
L. E. O.*	1	3.60	1.30	2.30	1.20	1.10	1.00	0.10	0.57
K. V. I.*	1	4.70	1.50	3.20	0.90	2.30	1.80	0.50	0.47
W. I. L.*	2	4.55	1.50	3.05	0.70	2.35	1.70	0.65	0.47
Average of 16 patients	*20*	*4.00*	*0.69*	*3.31*	*1.12*	*2.19*	*1.89*	*0.30*	*0.21*
Average normal values	†	*6.58*	*3.42*	*3.16*	*1.06*	*2.10*	*1.32*	*0.78*	*1.08*

*Starred patients are children.

†Data from the Harvard Plasma Fractionation Program[20], Table IX recalculated for the omission of fibrinogen.

TABLE IV. REPORTED VALUES OF SERUM PROTEIN FRACTIONS IN NEPHROTIC SYNDROMES AS OBTAINED BY ELECTROPHORETIC AND BY CHEMICAL ANALYSIS

AUTHOR	NUMBER OF PATIENTS	TOTAL PROTEIN (GM. %)	ALBUMIN (GM. %)	TOTAL GLOBULIN (GM. %)	ALPHA GLOBULIN (GM. %)	BETA PLUS GAMMA GLOBULIN (GM. %)	BETA GLOBULIN (GM. %)	GAMMA GLOBULIN (GM. %)	A/G RATIO
				Etiology: Glomerulonephritis					
Electrophoretic fractionation									
Longsworth and MacInnes[25]	2 (2 adults)	4.87	0.71	4.16	2.14	2.02	1.74	0.28	0.17
Luetscher[27]	4 (3 adults, 1 child)	3.70	0.73	2.97	1.13	1.84	1.60	0.24	0.25
Thorn and co-workers[20]*	6 (6 adults)	4.06	1.12	2.94	1.35	1.59	1.22	0.37	0.38
Wuhrmann and co-workers[16]	4 (4 adults)	4.15	1.47	2.68	1.16	1.52	0.94	0.58	0.55
Malmros and Blix[26]†	4 (4 adults)	5.44	1.52	3.92	1.60	2.32	1.26	1.06	0.39
Average electrophoretic data	*20 (19 adults, 1 child)*	*4.44*	*1.11*	*3.33*	*1.47*	*1.86*	*1.35*	*0.51*	*0.33*
Chemical fractionation (present study)									
Incomplete fractionations	*18 (5 adults,*	*4.24*	*1.29*	*2.95*	*1.07*	*1.88*	---	---	*0.44*
	13 children)	*4.52*	*0.71*	*3.81*	*1.00*	*2.72*	---	---	*0.19*
Complete fractionations	*16 (16 children)*	*4.00*	*0.69*	*3.31*	*1.12*	*2.19*	*1.89*	*0.30*	*0.21*
				Etiology: Amyloidosis					
Electrophoretic fractionation									
Malmros and Blix[26]†	1‡ (1 adult)	4.90	0.70	4.20	2.40	1.80	0.90	0.90	0.17
Luetscher[27]	1§ (1 adult)	3.59	1.31	2.28	0.83	1.45	0.51	0.94	0.57
Wuhrmann and co-workers[16]	1‖ (1 adult)	5.20	1.90	3.30	1.36	1.94	1.34	0.60	0.58
Average electrophoretic data	*3 (3 adults)*	*4.56*	*1.30*	*3.26*	*1.53*	*1.73*	*0.92*	*0.81*	*0.40*
Chemical fractionation (present study)	*2‖ (2 adults)*	*5.90*	*1.50*	*4.40*	*1.20*	*3.20*	---	---	*0.34*

*Total protein concentrations and schlieren patterns appear in the original article.[20] The relative concentrations of the various fractions were calculated by Loeb[18] from the schlieren patterns. The absolute values quoted in the table were calculated from the data of these two sources, with the omission of fibrinogen.

†The study of Malmros and Blix[26] was concerned only with patients with elevated sedimentation rates.

‡Etiology, chronic osteomyelitis.

§Etiology not given.

‖Etiology, chronic tuberculosis.

serum beta globulin of 2.09 Gm. per cent, while those with serum albumin concentrations above 1.0 Gm. per cent had an average serum beta globulin concentration of 1.36 Gm. per cent. With the hyperbetaglobulinemia is associated the hyperlipemia of the typical nephrotic patient since the beta globulin components which are increased in nephrosis carry large proportions of protein-bound lipid. The serum cholesterol values in such cases may be greatly elevated; they averaged 799 mg. per cent in one series of six patients.[20] In such sera, the presence of large amounts of lipid makes it difficult to determine the actual concentration of protein in the beta globulin fraction with electrophoretic methods since the lipids transported with the beta globulins increase the size of the beta globulin peak in the schlieren diagram; in this respect chemical fractionation is of distinct advantage, since protein alone is estimated.

Alpha globulin in the nephrotic patients exhibits neither the striking positive correlation with albumin values shown by gamma globulin nor the negative correlation shown by beta globulin. The group of children with albumin concentrations below 1.0 Gm. per cent had an average alpha globulin of 1.19 Gm. per cent, while those with higher albumin concentrations had an average alpha globulin concentration of 0.99 Gm. per cent. These differences are probably not significant, and it seems likely that the failure of alpha globulin values to change significantly may well be due to the fact that its two major components have different physiologic activities. Alpha-1 globulin is a component which resembles albumin in its general behavior and renal clearance and may appear in large amounts in nephrotic urine.[34] Alpha-2 globulin, on the other hand, more nearly resembles beta globulin in behavior and contains a certain amount of lipoprotein. A simultaneous decrease in the former and increase in the latter might well produce little net change in alpha globulin values.

It is difficult to compare the reported electrophoretic values in nephrotic patients (Table IV) directly with our values. Nineteen of the reported twenty electrophoretic values were obtained on adults while our values were obtained chiefly on children. However in the five adults included in our series of patients with nephritic nephrosis and in our two cases of amyloid nephrosis, the values obtained by chemical fractionation show less divergence from the mean electrophoretic values than do certain of the individual electrophoretic reports. From our data it appears likely that there may actually be some quantitative differences in the protein patterns of the adult and juvenile nephrotic patient. The average serum albumin is considerably higher in the adults, and from the preceding discussion one might expect this to be accompanied by beta globulin values which are not quite so high as in the juvenile nephrotic subject and by gamma globulins which are somewhat larger than in the younger group. On the whole, the review of electrophoretic data given in Table IV bears out these expectations and supports the view that the information gained by chemical fractionation is not essentially different from that obtained by electrophoretic analysis.

In the groups of patients other than the nephrotic subjects the average albumin values fell between 1.5 and 2.0 Gm. per cent (Table II). In the patients with reticulo-endothelial involvement and with hepatic dysfunction, the total

serum globulin and the beta plus gamma globulin concentrations averaged more than 1.5 Gm. per cent greater than those found in the average hospital patient. In the patients with gastrointestinal involvement and in the nephrotic patients total serum globulin and beta plus gamma globulin concentrations either were normal or were increased less than 0.75 Gm. per cent.

DISCUSSION

The application of chemical fractionation procedures to a large number of routine hospital determinations has confirmed, in general, the occurrence of hypoalbuminemia in those syndromes in which this finding previously had been reported by investigators who used electrophoretic analysis. In addition, hypoalbuminemia has been found in certain disorders which involve the reticuloendothelial system and which ordinarily produce more pronounced alterations in serum globulins than in albumin. Statistically, cases of the nephrotic syndrome accounted for over half of the patients in whom albumin concentrations of 2.0 Gm. per cent or less were found, and this syndrome accounted for all of the patients in whom albumin concentrations of 1.0 Gm. per cent or less were found.

The finding that hypoalbuminemia is not only most frequent but also most severe in the nephrotic syndrome has certain implications for the pathogenesis of nephrotic edema. It is clear, for example, that whatever factors other than hypoalbuminemia may be involved,[17, 18, 30-32] hypoalbuminemia must play a larger role in the pathogenesis of nephrotic edema than in the pathogenesis of any other systemic edema. The low albumin concentrations, the suggestion that the serum albumin of the nephrotic patient may come to consist chiefly of an albumin subfraction of large molecular size,[16, 18, 33, 34] and the absence of "compensatory" hyperglobulinemia appear satisfactorily to account for the greatly diminished plasma oncotic pressure of the nephrotic patient.[35-37] It seems possible that edema, in the nephrotic subject, increases tissue turgor to a point where increased resistance to loss of fluid from the capillary balances, to some extent, the increased tendency to lose fluid which is the consequence of hypoalbuminemia. In this way, nephrotic edema becomes a dynamic factor which makes possible the maintenance of a more or less adequate circulating plasma volume. It is not clear why, in the nephrotic patient, dynamic compensation for

TABLE V. SERUM PROTEIN FRACTIONATION PATTERNS IN PATIENTS WITH HYPOALBUMINEMIA

FOUND IN:	NEPHROTIC SYNDROME, GASTROINTESTINAL INVOLVEMENT	HEPATIC DYSFUNCTION, RETICULO-ENDOTHELIAL SYSTEM INVOLVEMENT	HYPOGAMMAGLOBULINEMIA WITH LYMPHATIC ATROPHY
Total protein	Usually low	Normal or high	Low
Albumin	In nephrotic patients may be below 1.0 Gm. %	Above 1.0 Gm. %	Above 1.0 Gm. %
Total globulin	Normal or elevated less than 1.0 Gm. %	Elevated more than 1.5 Gm. %	Low
Alpha globulin	Normal or slightly elevated	Usually normal	Normal
Beta plus gamma globulin	Normal or elevated less than 1.0 Gm. %	Elevated more than 1.5 Gm. %	Very low
A/G ratio	Usually below 0.5	Usually below 0.5	High

decreased plasma oncotic pressure should depend largely upon alterations in interstitial pressure, while in most cases "compensatory" hyperglobulinemia (relative or absolute) aids in compensating for decreased oncotic pressure.

In patients with hypoalbuminemia, the presence or absence of "compensatory" hyperglobulinemia, the severity of the hypoalbuminemia, and the alterations in beta plus gamma globulin concentration make possible a rough differentiation of three patterns of hypoalbuminemia (Table V). In the nephrotic subject and in patients with gastrointestinal involvement, the pattern is that of hypoalbuminemia with little "compensatory" hyperglobulinemia. In patients with hepatic dysfunction and with reticulo-endothelial involvement, albumin concentrations are not so low as in nephrosis, and globulin concentrations are significantly elevated. The pathologic physiology of these latter two groups of diseases strongly suggests that the hyperglobulinemia is in fact not "compensatory" but an effect produced by involvement of the globulin-forming tissue in the disease process. It is possible that the differences in pathologic physiology which are reflected in the findings of Table V may, on occasion, be employed as an aid to differential diagnosis.

CONCLUSIONS

Rapid chemical fractionation procedures which give results approximating those of electrophoresis have been applied to a study of 500 sera received for routine analysis.

Systematic comparison of the albumin values obtained by this method with Howe "albumin" values shows the latter to conceal both the frequency and magnitude of hypoalbuminemia.

Serum albumin concentrations of 1.0 Gm. per cent or less occurred only in ten patients with the nephrotic syndrome.

Serum albumin concentrations of 2.0 Gm. per cent or less occurred in an additional ten nephrotic patients and in fourteen other patients who were grouped into the general categories: severe gastrointestinal involvement, hepatic dysfunction, and diseases with involvement of the reticulo-endothelial system.

The increase in serum beta globulin and decrease in serum gamma globulin which has been reported in nephrotic adults has been confirmed in a group of sixteen nephrotic children. There is a marked correlation between the degree of hypoalbuminemia, the magnitude of increase in serum beta globulin, and the magnitude of decrease in serum gamma globulin.

Increases in total globulin or in alpha globulin were slight or absent in the patients with nephrotic syndrome or gastrointestinal involvement. In patients with severe hepatic dysfunction or reticulo-endothelial involvement, total globulin and beta plus gamma globulin were increased, on the average, more than 1.5 Gm. per cent.

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FAILURE OF ANTIRETICULAR CYTOTOXIC SERUM IN ARTHRITIS

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THE imposing list of diseases claimed by Bogomolets[1] and his co-workers to have been benefited by the antireticular cytotoxic serum (ACS) includes also acute rheumatism. It is to be regretted that the translators were not aware that in part of the European literature this term indicates rheumatic fever. That the latter was meant by the Russian authors is evident by the qualification that antireticular cytotoxic serum is contraindicated in patients who suffer from endocarditis and myocarditis.

The sensational press notices omitted the word acute, and both the public and physicians were fired with the expectation that a cure for all forms of rheumatism was discovered. A great clamor was raised, and when the serum was produced in this country it was immediately introduced into the therapy of various forms of arthritis.

Previous studies of the reticulo-endothelial elements in the synovial membrane and its role in the chemotherapy of rheumatoid arthritis[2-4] have made me aware of objections to the extended concept of the reticulo-endothelial system (RES) and the therapeutic application proposed by Bogomolets and his school. However, for one engaged in the practice of rheumatology an investigation of the effect of antireticular cytotoxic serum in rheumatic conditions became necessary in order to reach independent conclusions concerning its efficacy.

PREPARATION AND DOSAGE OF ANTIRETICULAR CYTOTOXIC SERUM

The methods of preparation and standardization of antireticular cytotoxic serum were described by Marchuk,[5] and modifications by American investigators[6,7] followed.

The majority of our patients received rabbit antireticular cytotoxic serum prepared in lyophilized form* and supplied in a combination package. One vial contained .4 ml. desiccated serum and another vial 4 ml. of physiologic saline solution. In order to discover hypersensitivity, a preliminary intradermal test was carried out with normal rabbit serum. Some patients were given rabbit and/or goat antireticular cytotoxic serum.†

Two schedules were used. One followed the original directions of Bogomolets. A course consisted of an injection of .05 ml. of antireticular cytotoxic serum followed after an interval of two or three days by .1 ml. and concluded with .15 ml. after the same interval.

The second method of administration was developed by Straus and associates. Injections were given twice weekly, starting with .02 ml. of antireticular cytotoxic serum. The dosage was increased by .02 ml. at each subsequent injection to a maximum of .2 or .25 milliliter. This dose was repeated up to six weeks, depending on the response of the patient.

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†Produced by Dr. Straus in the Department of Pathology and Research, Cedars of Lebanon Hospital, Los Angeles, Calif.

With both methods the series can be given several times after an interval of at least four weeks. Before each series the intradermal test with rabbit serum was repeated in order to discover if hypersensitivity had developed in the interval.

In some of our patients we reduced the initial dose to .01 ml. in order to avoid reactions. If there were marked reactions to rabbit serum, goat serum was substituted. If this also gave reactions, the treatment was discontinued. According to Bogomolets the use of antireticular cytotoxic serum is contraindicated in acute and chronic endocarditis, myocarditis, nephrosis and nephritis, bronchial asthma, and exudative tuberculosis.

It is recommended that injections be given subcutaneously.* The intravenous route was favored by some Russian authors. They (Kavetskiy[8]) applied various tests to determine the functional state of the reticulo-endothelial system and the effect of the antireticular cytotoxic serum upon it. Most of these tests are too complicated for clinical use; none is specific. Only the sedimentation tests and the hemogram were carried out on our patients. A decrease in sedimentation rate and an increase in the percentage of monocytes and lymphocytes is supposed to indicate an enhanced function of the reticulo-endothelial system.

Table I gives the results in sixty cases of the various types of rheumatic diseases.

TABLE I. RESULTS OF ANTIRETICULAR CYTOTOXIC SERUM IN SIXTY CASES

TYPE	NUMBER OF CASES	IMPROVED		NOT IMPROVED	WORSE
		MODERATELY	SLIGHTLY		
Rheumatoid arthritis	30	1	4	16	9
Osteoarthritis	20	2	4	12	2
Fibrositis	10	2	1	6	1
Total	60	5 (8.3%)	9 (15%)	34 (56.6%)	12 (20%)

RHEUMATOID ARTHRITIS

Thirty patients with rheumatoid arthritis received antireticular cytotoxic serum. They represented a cross section of the adult form of the disease, as seen in our clinic and private practice. Numerous peripheral joints were affected. Cases of Strümpell-Marie spondylitis and Still's disease (adolescent rheumatoid arthritis) were not included. Twenty patients were women; ten were men. The ages ranged from 23 to 63 years. The average age was 45.3 years. The duration varied from two to twenty years; the average duration was five and one-half years. The arthritis was mild in three patients, moderate in thirteen, and severe in fourteen. Twenty-three patients had received previously one or more courses of gold therapy and fourteen of these have had significant objective improvement or remission, lasting from one to five years.

Fourteen patients received one course of the short or long series of antireticular cytotoxic serum. Sixteen patients were given from two to six series of injections. In the first group the course was not repeated because of local or generalized adverse reactions. During the period of antireticular cytotoxic serum therapy, supporting nutritional, physical, corrective, and analgesic treatment was continued.

The results were evaluated after conclusion of the treatment and at a check-up six months to a year later. One patient showed moderate and four slight subjective and objective improvement. The amelioration lasted not more than six weeks after completion of the treatment. Sixteen patients showed no im-

*By Wyeth Incorporated and Dr. Straus.

provement and nine became worse. The duration of the disease in the moderately improved patient was thirteen years and varied in the three slightly improved from nine to fifteen years. Among those not improved there were four cases of only two years' duration and four of less than four years' duration. Therefore responses were not better when the disease was of shorter duration or in a less advanced stage.

In a control group of 220 patients who were treated with various nonspecific measures alone, about 15 per cent had marked subjective and objective improvement. In 465 patients with rheumatoid arthritis treated by gold therapy, immediate marked objective improvement was achieved in over 52 per cent. Antireticular cytotoxic serum therefore was far less efficient than gold therapy and even nonspecific measures.

The following is evidence that the poor results with antireticular cytotoxic serum were not due to intractability of the material: twelve patients received gold therapy after antireticular cytotoxic serum had failed; of these seven had at least a moderate objective improvement. Four others were improved with other measures.

The initial sedimentation rate varied from 15 mm. to 80 mm. after an hour, Westergren's method. There was no significant change in the rate after antireticular cytotoxic serum therapy, even in the five instances associated with some degree of improvement. In patients who had severe reactions the sedimentation rate rose. No marked shift in the hemogram occurred. The number of injections and the length of treatment did not have a significant influence on the results. Three of the slightly improved patients received one to two series of three injections. On the other hand, six of those who got worse received multiple courses with as many as fourteen injections per series.

OSTEOARTHRITIS

Twenty patients with osteoarthritis were treated. All had typical clinical and roentgenographic evidence of the affection. Fifteen had involvement of the spine either alone or associated with osteoarthritis of the hips, hands, and knees. Heberden's nodes were present in four persons. One patient had involvement of the shoulders. The ages ranged from 42 to 73 years; the average age was 55.8 years. Eighteen patients were women and two were men. This unusual distribution resulted from the prevalence of women among our patients. The duration of presenting symptoms was relatively short, from two weeks to three years. The average duration was one and a half years. The sedimentation rates were normal or only slightly elevated in several cases.

Under antireticular cytotoxic serum therapy only two patients were moderately improved and three slightly; twelve were unimproved and three got worse. One of the moderately improved patients relapsed one month after the treatment. Eleven patients unimproved by antireticular cytotoxic serum were given physical, nutritional, orthopedic, and climatic therapy. Of these, three were unimproved; eight had significant improvement. Of those significantly

improved, six remained so at least for a year. These results clearly show antireticular cytotoxic serum inferior to nonspecific measures commonly used in osteoarthritis.

FIBROSITIS

Ten patients were treated. The presenting symptoms were pain and stiffness of the various muscle groups, especially on arising and after remaining in one position. They were ameliorated after activity and warming up. The lower back, the shoulders, and the arms were involved. There was tenderness and spasm of the affected muscles. Fibrositic nodules could be palpated in some instances. The roentgenograms showed the joints to be normal. The sedimentation rates were normal or slightly increased. The ages varied from 32 to 50 years, with an average age of 43 years. The duration of the symptoms was from six weeks to five years; average duration, two years. Two patients were moderately improved; one was slightly improved; six were unimproved; and one got worse. One patient with fibrositis of the right forearm, which was moderately improved, had a severe local and systemic reaction, with fever, malaise, and headache. A week after the reaction had subsided the patient had a recurrence and spread of the symptoms to the left forearm and lower back. Granted that this group was more chronic and stubborn than the majority of cases, the results of the antireticular cytotoxic serum in fibrositis were disappointing.

REACTIONS

Of the sixty patients, thirty-seven (62 per cent) had reactions; of these, fourteen (38 per cent) were mild, nine (24 per cent) were moderate, and fourteen (38 per cent) were severe. There was no significant difference in the degree of reactions in the various types of rheumatic diseases treated: 56 per cent of patients with rheumatoid arthritis, 70 per cent with osteoarthritis, and 60 per cent with fibrositis had reactions. Severe reactions occurred in 23 per cent with rheumatoid arthritis, 20 per cent with osteoarthritis, and 20 per cent with fibrositis. Mild reactions consisted of inflammation and tenderness of an area of several inches surrounding the point of inoculation. In moderate reactions the inflammation spread over a large part of the arm. In severe reactions it involved the whole arm to the elbow. The skin and subcutis became edematous, but there was no suppuration. In one case of rheumatoid arthritis an effusion in the olecranon bursa occurred. The reactions started in six to twenty-four hours after injection. With the severe reactions there were low-grade fever of one to three days' duration, joint pain, malaise, headache, and lymphadenitis. In sixteen patients pruritus and urticaria occurred; in six, these reactions were general. Eczema under the axillae and thighs occurred in a patient with rheumatoid arthritis. In one patient with osteoarthritis, ecchymosis and purpuric spots developed around the puncture points and over the forearm. One patient had marked palpitation during treatment. Three osteoarthritic patients developed angioneurotic edema. In two patients the eyelids and in one the lips were involved for several days to two weeks.

The reactions were nonspecific manifestations of hypersensitivity to foreign proteins and/or allergy.

During and after the course of treatment, a definite increase in hypersensitivity to antireticular cytotoxic serum occurred in some allergic patients. This is illustrated by a female patient with rheumatoid arthritis who developed urticaria in the first course after .15 milliliter. A second series was started after six weeks with only .01 ml. and she developed a local reaction and urticaria after the second injection of only .02 milliliter. Previous to antireticular cytotoxic serum administration the patient had a severe urticaria after massive intramuscular doses of penicillin and beeswax in oil.

The majority of reactions occurred with increasing dosages of antireticular cytotoxic serum. When the Bogomolets schedule of three injections was used, the patients usually tolerated the frst injection of .05 ml. but reacted to the second (.1 ml.) or third (.15 ml.) injection. In the longer series, starting with .02 ml., most of the reactions occurred at the middle or end of the course, when the dosage approached .1 or .2 milliliter. Two patients treated elsewhere with long series of injections manifested acute inflammation of the previously normal thumb and wrist joints. Prolonged series of injections with very short intervals were given for one or more years by some physicians. If antireticular cytotoxic serum should have a cumulative effect, the prolonged therapy may act similarly to a blocking of the reticulo-endothelial system. In experimental animals, Emerson, Ewing, and Thomas[9] produced severe macrocytic anemia by high dosage of antireticular cytotoxic serum.

DISCUSSION

Bach[10] in 1945 reported forty-eight patients with different types of arthritis treated by antireticular cytotoxic serum. Thirty-five of these had rheumatoid arthritis. Rogoff, Freyberg, Powell, and Rice[11] in 1947 published results on the treatment with antireticular cytotoxic serum of twenty-nine patients with

TABLE II. RESULTS OF ANTIRETICULAR CYTOTOXIC SERUM IN RHEUMATOID ARTHRITIS

AUTHOR	YEAR	NUMBER OF PATIENTS	OBJECTIVE IMPROVEMENT		RELAPSED AFTER WEEKS TO 12 MO.
			CASES	PER CENT	
Bach[10]	1945	35	3	9	2
Rogoff and co-workers[11]	1947	29	3	10	-
Boots and co-workers[12]	1947	34	7	20	2
Kling	1948	30	5	16	5
Total	1945-1948	128	18	14	9 (50%)

rheumatoid arthritis. Eight patients with rheumatoid spondylitis (Strümpell-Marie type) were included in this series. Boots, Coss, and Ragan[12] in 1947 analyzed results in thirty-four patients with rheumatoid arthritis. These authors concluded that the results of antireticular cytotoxic serum were discouraging or inconclusive.

Table II summarizes their combined results and my own in rheumatoid arthritis. Of 128 patients treated, eighteen (14 per cent) had some degree of objective improvement. In the majority this improvement was only moderate or slight. In over 50 per cent relapses occurred after a short period. As

pointed out previously, an equal or better percentage of improvement was obtained by nonspecific measures.

Moreover, Rogoff and co-workers[11] gave to a control group of fourteen patients with rheumatoid arthritis normal rabbit serum alone. Two patients (14 per cent) showed objective improvement and one had subjective improvement. The results equalled those obtained by antireticular cytotoxic serum. This suggests that any benefit may be due to a nonspecific serum effect rather than to the specific influence of antireticular cytotoxic serum. Bach used antireticular cytotoxic serum prepared in Bogomolets Institute and sent to England. Straus[13] recently pointed to the possibility that this serum was inactivated in transit. He found a loss of antibody titer below significant levels in antireticular cytotoxic sera which he had received from Russia. However, this does not explain the equally poor results of the other authors mentioned who worked with antireticular cytotoxic serum produced in the United States and tested and approved in Dr. Straus' laboratory.

CRITICISM OF BOGOMOLETS' CONCEPT OF THE RETICULO-ENDOTHELIAL SYSTEM

Rheumatoid arthritis should be the most likely type of arthritis to respond favorably to antireticular cytotoxic serum, if Bogomolets' concept is true. Although its specific etiology is unknown, inflammatory reactions are present in the synovial tissues, in the subchondral bone marrow, in the lymph nodes, muscles, tendons, perivascular and perineural tissues, in the skin, and in other organs. Some investigators regard the disease as a systemic hyperergic reaction, especially of the mesenchymal tissues, closely related to rheumatic fever. In the latter, according to Strazhesko,[14] antireticular cytotoxic serum is beneficial in the second, hyperergic, stage. Therefore the failure of antireticular cytotoxic serum in rheumatoid arthritis is very disappointing. It was pointed out that Bogomolets and co-workers are not directly responsible for the use of the antireticular cytotoxic serum in these forms of chronic arthritis.

However, the greatly expanded concept of the reticulo-endothelial system, which, besides the macrophages and histiocytes in different organs and subcutaneous tissues, includes all unformed connective tissue and its derivatives, such as osteoid, cartilaginous, and synovial tissue, lends itself to the assumption that a general remedy has been discovered for treatment of every disease which is located in organs of mesodermal origin, for instance all forms of arthritis.

Against such unwarranted generalization it should be stressed that the function of the reticulo-endothelial system, which is established beyond a doubt, is phagocytosis, storage and digestion of particulate living or dead matter, such as bacteria, cells, fat, and colloidal dyes. The evidence of other important functions, such as antibody formation, is controversial.

MacMasters and Hudack[15] and Ehrich and Harris[16] demonstrated by a series of ingenious experiments that the antibodies are elaborated within the lymphocytes and also possibly by the plasma cells. Under ordinary conditions these cells do not phagocytose or take up selectively vital dyes. The inclusion

of these cells in the reticulo-endothelial system therefore is not justified. Kass[17] proved that the lymphocytes produce the normal gamma globulins and the immune globulins. Pomerat[18] has shown that the globulins are the effective fraction of antireticular cytotoxic serum. The antigen is extracted from four parts of spleen and one part of bone marrow. Therefore it contains a large portion of lymphocytic extract and the potency of antireticular cytotoxic serum as an antibody is at least partly due to the lymphocytic fraction. Jaffé[19] rightly pointed out: "It is not clear why the authors who have prepared or used extracts of the spleen identify the substances obtained from this organ with the reticulo-endothelium since the spleen is composed not only of reticulo-endothelial cells but also of other structures."

Applied to the rheumatic diseases, the objections can be summarized as follows.

First, it is not proved that the reticulo-endothelial system is depressed in any type of these diseases.

Second, the role of the reticulo-endothelial system in pathogenesis and therapy has not been definitely established. In the rheumatic diseases presented in this material and reviewed from the literature, the results of antireticular cytotoxic serum have been discouraging.

Third, it is doubtful that antireticular cytotoxic serum is a true antireticular serum.

The consistent efforts of Bogomolets and his school have stimulated valuable research. However, until accurate and practical methods of evaluation of the reticulo-endothelial system and its role in a given disease have been elaborated and until a firm basis of action and efficiency of antireticular cytotoxic serum has been established and possible adverse effects have been eliminated, its introduction into general practice, especially in the therapy of rheumatic diseases, should not be encouraged.

SUMMARY AND CONCLUSIONS

The therapeutic effects of antireticular cytotoxic serum were studied in sixty patients with different types of chronic rheumatic diseases. On the basis of our material and a review of the literature it is concluded that there is no indication for antireticular cytotoxic serum in the treatment of osteoarthritis and fibrositis, which are ameliorated as a rule by physical, orthopedic, and drug therapy. In rheumatoid arthritis and Strümpell-Marie spondylitis, which are so serious and often resistant to treatment, a trial of antireticular cytotoxic serum, on the basis of an occasional significant improvement, may be justified.

Important objections to the concept of the extended reticulo-endothelial system have been pointed out.

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THE EFFECT OF SUCCINATE IN MESCALINE HALLUCINATIONS

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INTRODUCTION

INVESTIGATIONS by Quastel and Wheatley[1, 2] have indicated that the oxidation of succinate is not inhibited by the presence of barbiturates and certain of the narcotic amines as is that of glucose, lactate, and pyruvate. Following this very suggestive conclusion, Soskin and Taubenhaus[3] proposed the use of sodium succinate as an antidote to barbiturate poisoning in which the succinate would serve as an oxidizable substrate until the barbiturate was eliminated from the body. Other investigators[4-9] are divided in opinion as to the antidotal effect of succinate in barbiturate hypnosis. Comparative experiments by DeBoer[10] concerning the diuretic effect of succinate and sucrose indicate that the decrease in sleeping time produced by succinate in barbiturate hypnosis may be correlated with the degree of diuresis produced.

Succinate also has been found to be of considerable experimental interest in the treatment of diabetic acidosis,[11] in protection against poisoning by dithiols,[12] and in the place of salicylates in the treatment of rheumatic fever.[13]

In spite of this new interest, the possibility of using succinate in the treatment of narcotic amine depression has not been investigated. In particular, we have become interested in the possible effects of succinate on the very striking visual hallucinations produced by mescaline (3,4,5-trimethoxy-β-phenyl ethylamine). Mescaline effects have long held the interest of experimental psychiatrists and pharmacologists through the possible insights they may give into some aspects of the mechanism of hallucinosis. The literature on mescaline is vast, with a particular emphasis upon visual effects. Klüver,[13] in a comprehensive review, outlines the visual effects characterizing the progressive intoxication of human subjects from a single dose of mescaline sulfate. These comprise a set of phenomena which are more or less constant for all individuals and include the development of (1) grating, lattice, fretwork, filigree, honeycomb, or chessboard, (2) cobweb, (3) tunnel, funnel, alley, cone, or vessel, and (4) spiral.

Many other phenomena are, on close examination, nothing but modifications and transformations of these basic forms. The tendency toward geometrization, as expressed in these form constants, is also apparent as the intoxication progresses. The forms are frequently repeated, combined, or elaborated into ornamental designs and mosaics of various kinds. The elements constituting these forms, such as squares in a chessboard, often have boundaries consisting of geometric figures.

These developments are accompanied by an equally vivid development of brilliant colors incorporated into the changing designs. The compounding of

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the designs leads to mental images of formed objects—faces, chairs, mountains, groups of people, and so on.

In addition to the visual phenomena, certain physiologic effects will be mentioned later in the discussion which may have some bearing on the results of this study. The foregoing general outline regarding the development of visual effects is important for orientation to the chronological order and depth of the effects produced in the following experiments.

EXPERIMENTAL

Experimental procedures were carried out in two directions: (1) in vitro studies concerning the effect of mescaline sulfate on rat whole brain respiring on glucose, lactate, pyruvate, and succinate, and (2) in vivo studies on normal human subjects. The method followed in the in vitro studies was that of Quastel and Wheatley[2] and involved manometric measurements on chopped, mixed, whole rat brain tissue in Krebs-Ringer and (M/15) phosphate medium at a pH of 7.4. The mescaline was made up in Krebs-Ringer-phosphate media and neutralized to pH 7.4 before addition to the brain. The brain suspension (100 to 200 mg.) was allowed to respire in air in the Warburg flasks in the presence of the drug until the uptake of oxygen had fallen to about 75 per cent of its

TABLE I

SUBSTRATE	OXYGEN UPTAKE* IN MM.^{3} AT 37° C. FOR 0.5 GM. RAT BRAIN FOR 2 HR.		PER CENT DECREASE DUE TO MESCALINE
	WITHOUT MESCALINE	WITH MESCALINE (0.1%)	
Saline	300	290	3.0
Glucose 0.025%	620	390	59.0
Sodium lactate 0.025%	730	480	52.0
Sodium pyruvate 0.025%	790	450	53.0
Sodium succinate 0.025%	860	852	1.0

*Each uptake expressed in the table is the average result of seven flasks.

initial value, at which time the substrate was added. This initial period was usually about two and one-half to three hours. Readings were taken for two hours after substrate addition, and the per cent decrease in oxygen consumption by 0.5 Gm. of rat brain due to exposure to mescaline at a final concentration of 0.1 per cent in the presence of glucose, lactate, pyruvate, and succinate was noted. These results are reported in Table I.

Subjects for the in vivo experiments were sophomore medical student volunteers. These individuals were given doses of 150 to 480 mg. of mescaline sulfate intramuscularly and the hallucinations were allowed to develop until the designs observed were of great complexity and the colors of vivid and rapidly changing hues. Then sodium succinate (20 per cent in sterile water) was injected to the extent of 3 to 6 Gm. intravenously, and the effects of the succinate on the hallucinations were recorded. The actual procedures were carried out in the Psychopathic Hospital, Department of Psychiatry, where all volunteers were

kept under careful observation at least twelve hours following the acute phases of the experiment.

The results of these experiments are outlined in time sequence of the effects, the drug being administered intramuscularly (gluteally) in physiologic saline solution. The acute phases of the experiment were carried out in a dimly lighted quiet room with the subject reclining. Subjects were advised to keep their eyes shut and report frequently, with prompting if necessary, the progress of the visions and any other symptoms involved during the course of the experiment. The experiments summarized in the following outline consist of: (1) three trials illustrating individual sensitivity to mescaline throughout the acute course of mescaline intoxication (Experiments 1 through 3), and (2) four trials illustrating the effect of sodium succinate given intravenously near what appeared to be a peak effect of mescaline vision development (Experiments 4 through 7). Only those subjects showing a relatively high sensitivity as evidenced by profuse visions of great complexity and color were chosen for the succinate administration.

EXPERIMENT 1.—20-year-old male subject; 132 pounds; B.P., 134/74; pulse, 108/min.

2:12 150 mg. mescaline sulfate intramuscularly.
2:55 First hallucinatory phenomena. Black and red horizontal lines in definite sequence.
2:58 Lattice work of snow crystals.
3:00 Square building-like objects in all colors in one plane.
3:08 Swarms of little fish-like objects.
3:15 Perfectly drawn black circles on a yellow background.
3:20 Purple snails with impression of the sky turned upside down.
3:25 Looking down on a row of tents hung over a line with scraps of colored glass scattered about.
3:30 Looking up at sky as through apex of a cone. Bright waves of color.
3:33 "All different colored stars."
3:35 Little doll-like people coming out of a hall in every direction.
3:40 "Different colored balls in tinsel going crisscross, bumping and sticking together."
3:42 Increasing hesitancy to answer questions. Fancy buildings, lacework bricks, "Just floating through."
3:45 One woman with a dog, man tips hat, wind is blowing and woman is gone. Smiles, hesitates—"Everything tickles me"—"Most of the time I feel I'm just watching but once in a while I'm taking part."
4:00 Big smile. Towers, not real towers, lacey like Eiffel tower. "When I see things, I want to keep them but can't."
4:10 "I feel big—big like a man with big hands that can break real big things." Negativism is extreme and subject answers questions with great hesitation.
4:30 Chicken coop with wire around—multicolored background. "I see spots, color spots." "You bother me." "I want to be left alone."
4:35 Things getting darker. "I'm hungry."
4:40 Moved to convalescent ward and a meal.
5:30 Still a few dull colors and simple designs.
8:30 Almost all effects except a slight intoxicated feeling have passed off. Pushing on eyeballs gives some bright colors.
9:00 The next morning. Feels fine. Still some slight color intensification upon pushing on eyeballs.

EXPERIMENT 2.—23-year-old male subject; 140 pounds; B.P., 119/29; pulse, 90/min.

10:20 210 mg. mescaline sulfate intramuscularly.
10:27 Fingers feel cold.
10:28 A shiny peach-colored ball in center of visual field.

10:34 A lattice work of lines (when pressing on eyeballs).
10:45 Slight nausea and dizziness. Sees nothing without pressing on eyeballs.
11:00 Sees color background start to fill the field, but it passes away when he tries to observe it more closely.
11:08 A dull green pattern which is greatly intensified by pressing on eyeballs.
11:20 "Feel bulbous." Feels chair and arm to be out of proportion. One side of body vastly larger than the other.
11:30 Some hesitancy in answering questions with a tendency to gesture and shake head in answering rather than talk.
12:00 No visual effects without pressing eyeballs.
12:30 Feels room is distorted and hallway is funnel-like but no color or design effects.
2:00 Feels fairly normal.

EXPERIMENT 3.—25-year-old male subject; B.P., 128/68; pulse, 88/min.

1:27 300 mg. mescaline sulfate intramuscularly.
1:40 Slight vertigo.
1:50 Fullness in head with tingling in lips and around mouth.
2:00 Legs feel unusually heavy. Visual field somewhat "lighter" but no effects except a dull flicker when pressing on eyeballs.
2:15 An additional 60 mg. mescaline sulfate intramuscularly.
2:40 No effect even when pressing eyes.
2:50 Still no visual effects.
4:20 Still no visual effects. Pupils dilated somewhat.

Succinate Experiments.—

EXPERIMENT 4.—Same subject as in Experiment 1.

1:25 220 mg. mescaline sulfate intramuscularly.
1:50 Predominantly white and black line patterns over field changing to subdued green and blue on white. Colors alternating quite rapidly.
1:55 Lacework—white on black with color in the background getting progressively clearer.
2:00 Small objects, like letters, revolving on lacework background, then little forms of bright red and orange making up a wheel.
2:10 Many rings and various abstract patterns getting larger and brighter.
2:30 Now many bright colored, multiple formed objects floating in air.
2:45 Brilliant scenes of formed objects like arm chairs floating across valleys.
(5 Gm. of sodium succinate injected intravenously, slowly, into arm vein.)
There is an almost immediate dulling of color and the formed objects are replaced by simpler patterns which are on a brownish background.
2:55 A brownish haze with no patterns. The subject is now fairly talkative and exclaims about the rapid change.
3:30 The visual field seems lighter, but no designs or color even upon pressing on eyeballs.
3:45 Pressing on eyeballs reveals some fretwork of dull brown.
4:00 The fretwork design with some bright flashes seems more complicated but is clear only when pressing on eyes.
From this time on, light colors appear as background and develop with design mainly of the lined fretwork type. These effects gradually pass off by 9:00 P.M.

EXPERIMENT 5.—26-year-old male subject; 175 pounds; B.P., 120/74; pulse, 90/min.

1:25 480 mg. mescaline sulfate intramuscularly.
1:35 Six-spike snowflakes clustered around a luminous ball of green-yellow in the center.
1:45 Bright yellow circle like a doughnut. The background is marked off in shiny blocks.
2:00 Feels "high," drunk. Brilliant colors everywhere. Feels very much disoriented in space.
2:40 Complicated designs and rapidly changing colors. The subject is very hesitant at this time in answering questions.
(6 Gm. of sodium succinate injected intravenously.)

2:50 "Color and design are gone except for dark blue-black background."
3:00 A few dark blue lines on dark background. Intoxication is passing off. The subject talks freely with no hesitancy. Now feels somewhat euphoric.
3:30 Still no color or design but feels a little euphoria again.
4:00 Feels somewhat numb and euphoric with a light color-tinged visual field with definite, simple, dull colored fretwork designs.
7:00 Euphoria has passed off. Very sleepy and exhausted.

EXPERIMENT 6.—22-year-old male subject; 131 pounds; B.P., 121/80; pulse, 85/min.

1:43 270 mg. mescaline sulfate intramuscularly.
1:50 Feels a slight "headiness" that comes and goes.
2:30 Dull flashes of light with background of green fading to purple.
2:45 A whirling design like a fan with violet spider webs.
2:50 Colors brighter with much red and green and occasional designs in orange or yellow.
2:55 People in silhouettes piling on top of each other on a background of Indian designs. A bright sun appears and spirals of bright yellow.
3:10 Brilliant patterns with silhouette figures in all colors.
(3.5 Gm. of sodium succinate intravenously.)
3:20 Colors have blurred and show mainly dull browns and greens on a black background.
3:35 Visual field is still dull with simple fretwork design on dark background only upon pressing eyeballs.
4:00 Feels fairly normal with color in background only on pressing.
5:00 Color is returning with drunkenness and "high" feeling. Colors quite vivid but patterns simple when pressing eyeballs.
6:00 Still "high" with some color, mostly dull browns and greens or blues in background. Color brightens when pressing eyeballs.
8:00 All color passed off with only some general visual field green or brown.

EXPERIMENT 7.—Same subject as in Experiment 5.

1:45 480 mg. mescaline sulfate intramuscularly.
1:50 Gray snowflakes in black and white mosaic pattern.
1:55 "Exhausted like at end of the day."
2:00 A little nausea.
2:10 Black and gray-white background with pin points of yellow and green. Color fading "in and out."
2:30 Design is increasing in complexity with slight increase in color. Fretworks, cobweb designs "melting in and out."
2:40 Progress in complexity with many details, various parts of visual field show isolated "independent" activity and development with effect of third dimension and perspective.
3:00 A considerable increase in color with many formed objects—people, animals in constant movement with a brick-colored mosaic background.
3:30 Much beautiful coloration and formed objects in three dimensions.
(5 Gm. of sodium succinate intravenously.)
3:35 Faces of people and formed objects rapidly "melt" and "dissolve" with an almost immediate cessation of color effects.
3:40 No patterns or color even when pushing on eyeballs. Feels fairly normal with a little euphoria.
4:00 Some vague design of a simple mosaic type when pushing on eyeballs.
4:30 Still some simple design and a little color when pushing on eyeballs.
5:30 Feels quite normal but sleepy and hungry.
8:00 Sees vague gray on black, simple lattice work when pushing on eyes in a darkened room.

Miscellaneous Effects Observed in Some or All of the Subjects Which Are Caused by Mescaline and Which Are Antidoted Partially by Succinate.—Abolition of cremaster reflex; abolition of bladder filling sense; increased knee jerk reflex; depression and decrease in cooperation, with hesitancy of speech; space and time distortion; depression of respiration.

Effects Not Antidoted by the Succinate.—Dilatation of the pupil; hunger; tightness across the face.

DISCUSSION OF RESULTS

The in vitro experiments carried out on rat brain illustrate and confirm in essence the information obtained by Quastel and Wheatley that oxidations by brain tissue are inhibited less by mescaline when respiring on succinate than when utilizing glucose, pyruvate, or lactate. It should be pointed out, however, that these inhibitions are obtained only if the brain is incubated with the drug in the absence of substrate for the initial two and one-half to three hour period. None or very little inhibition was obtained in this laboratory when the substrate was added at the beginning even in the presence of vastly larger quantities of mescaline (up to 0.5 per cent). In light of the fact that relatively small doses of mescaline, considering the total body weight of human subjects, produce profound effects within thirty to sixty minutes after the intramuscular injection, it is difficult to correlate the conclusions of the previous investigators regarding the in vitro effects of mescaline with the rapid, very dramatic effect which it produces in relatively small doses in vivo. Nevertheless, the implication of an antidotal effect by succinate, in greatly decreasing the complexity of designs and color, was strikingly demonstrated. The immediate effect of succinate seems to be the complete cessation of hallucinosis in some instances and in others a decided regression of the visions to an earlier, simpler design with great loss in color intensity.

That the effect of succinate in lessening the hallucinosis can be due to a diuretic effect seems also unlikely since the succinate effect is so immediate. In addition, after thirty to sixty minutes the succinate effect seems to lessen, and color and design again appear, though still much less profuse than before the succinate. This would seem to indicate the presence of still toxic amounts of mescaline, making an antidotal hypothesis based upon a diuretic action seem untenable. Possibly a considerable part of the injected succinate has been utilized by other cells of the body in thirty to sixty minutes.

Also observable was an antidotal effect of succinate with regard to the general demeanor and physiologic responses of the subjects. Thus the decrease in cooperation and hesitance in speech observed were replaced after succinate by a more normal attitude and readiness to talk. The bladder filling sense returned and there was some lessening of the knee jerk reflex toward the normal. Respiration was increased by succinate, as was observed by others.[14, 15] Regardless of the mechanism, the effects seem most striking as described by one subject immediately after injection of succinate, "It was as if the colors and designs were being washed away."

SUMMARY

In vitro and in vivo experiments with mescaline sulfate were carried out. The in vitro experiments confirm the observations previously witnessed regard-

ing the lack of inhibitory action of mescaline on brain respiring on succinate and the definite inhibition of brain respiring on lactate, pyruvate, and glucose. In vivo experiments in human subjects demonstrate an antidotal effect of succinate on mescaline visions with regard to a decrease both in complexity of design and intensity of color. A consideration of the relatively large amounts of mescaline required to suppress brain respiration in vitro as compared with that required for the production of its in vivo effects, however, leaves some question as to the relation of the in vitro suppression to the process of hallucinosis.

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ABSORPTION, DISTRIBUTION, AND RENAL EXCRETION OF MANDELAMINE (METHENAMINE MANDELATE)

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MANDELAMINE, a urinary tract antiseptic now in clinical use, has been the subject of recent investigations in which it was reported[1, 2] that Mandelamine, streptomycin, and sulfathiazole are approximately equal in their activity against organisms which commonly invade the urinary tract. It was found that organisms develop resistance to sulfathiazole and streptomycin rapidly and to a remarkable degree, but, in contrast with these findings, resistance to Mandelamine did not appear at all. In order to gain further insight into the properties of Mandelamine, the following pharmacologic studies were undertaken.

EXPERIMENTAL

Chemical Methods.—Forty cubic centimeters of 0.1N sulfuric acid were added to solutions of Mandelamine (50 to 200 mg. per cent) and the solutions were steam distilled at a rate to produce 40 c.c. of distillate in sixteen minutes. The distillate was analyzed for formaldehyde by means of Deningès' colorimetric method.[3] Two hours were required for full color development. Final readings, measured in a photoelectric colorimeter at 5,800 Å. units, were compared with a reference curve prepared by using standard solutions of formaldehyde. (One gram of formaldehyde is equivalent to 1.62 Gm. of Mandelamine). The sixteen-minute distillation was selected because approximately 50 per cent of the Mandelamine is hydrolyzed to formaldehyde in this conveniently short period of time. Under these conditions analyses of Mandelamine solutions (3.12 to 200 mg. per cent) gave an average recovery of 53 per cent (Table I). Results are, therefore, divided by .53.

The method is directly applicable to urine, but application to blood requires a protein-free filtrate prepared according to the Haden modification of the Folin-Wu procedure. Mandelamine added to whole blood in concentrations of 50 to 200 mg. per cent was recovered as shown in Table II. The limit of sensitivity of the method, 0.6 mg. per cent, corresponds to a concentration of 6 mg. per cent in whole blood. In actual practice more reliable results were obtained when blood concentrations were 20 mg. per cent or above.

Creatinine was determined according to the Folin-Wu colorimetric procedure[4] modified for photoelectric measurement at 5,300 Å. units.

Distribution of Mandelamine in Whole Blood.—Mandelamine (25 to 200 mg. per cent) was added to heparinized dog blood and the mixture was shaken gently for thirty minutes. Samples of whole blood and plasma, analyzed for their Mandelamine content, gave the data (expressed in terms of blood and plasma water) shown in Table III. In order to approach physiologic conditions more adequately, the drug was injected intravenously into dogs, and samples of whole blood and plasma were then analyzed as before. The data indicate that the drug readily penetrates the red blood cell and distributes itself in proportion to the water content of whole blood. These experiments are of interest in con-

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TABLE I. RATE OF HYDROLYSIS OF MANDELAMINE

MANDELAMINE ADDED TO URINE (MG. %)	PER CENT CONVERSION IN 16 MIN.
3.12	54.4
12.5	53.6
50.0	51.3
200	52.9
Mean	53.0

TABLE II. RECOVERY OF MANDELAMINE ADDED TO WHOLE BLOOD

MANDELAMINE ADDED (MG. %)	MANDELAMINE RECOVERED (MG. %)	PER CENT RECOVERY
200	201.3	100.7
198	210.5	106.3
198	210.5	106.3
198	208.1	105.1
198	208.1	105.1
95.2	90.4	95.0
95.2	90.4	95.0
49.8	50.8	102.0
49.8	50.2	100.8
49.8	48.4	97.2
50.0	50.7	101.4
Mean		101.4 ± 1.3

TABLE III. PLASMA-ERYTHROCYTE PARTITION OF MANDELAMINE (DOG)

PROCEDURE	AMOUNT ADDED OR INJECTED	MANDELAMINE RECOVERED (MG. %)		P/B
		PLASMA/0.92	WHOLE BLOOD /0.79	
Mandelamine added to whole blood	24.8 mg. %	29.6	28.6	1.03
	118.8 mg. %	154.3	154.4	1.00
	118.8 mg. %	132.6	139.2	0.95
	200.0 mg. %	289.1	268.3	1.08
	200.0 mg. %	220.6	217.7	1.01
	200.0 mg. %	254.9	243.0	1.05
Mandelamine injected intravenously	750.0 mg./kg.	80.2	78.9	1.02
	750.0 mg./kg.	88.0	87.3	1.01
	750.0 mg./kg.	73.7	71.3	1.03
	250.0 mg./kg.	37.5	33.2	1.13
	250.0 mg./kg.	29.1	26.8	1.09
	250.0 mg./kg.	37.5	33.2	1.13
Mean				1.04 ± 0.05

nection with the following studies of the renal excretion of Mandelamine. It may be noted here that the erythrocyte membrane has been employed rather than the cellophane membrane frequently used in studies of protein binding.

Renal Clearance and Distribution of Mandelamine in the Dog.—Four mongrel, male dogs (weighing 7 to 14 kilograms), maintained on a standard ration, were fasted overnight before each experiment. At the outset the dogs were given 300 to 500 c.c. of water by stomach tube, and light anesthesia was induced by intravenous injection of 60 per cent of the anesthetic dose of Nembutal. Creatinine (150 mg. per kilogram) was injected subcutaneously, and ten minutes later additional creatinine was given both subcutaneously (75 mg. per kilogram) and intravenously (75 mg. per kilogram). Mandelamine (250 to 750 mg. per kilogram) was administered intravenously thirty minutes after the creatinine.

TABLE IV. RENAL CLEARANCE AND DISTRIBUTION OF MANDELAMINE

DOG	WEIGHT (KG.)	MANDELAMINE INJECTED (MG.)	TIME (MIN.)	CUMULATIVE RECOVERY IN URINE (MG.)	PLASMA CONC./0.93	MANDELAMINE CLEARANCE (C.C./MIN.)	CREATININE CLEARANCE (C.C./MIN.)	CLEARANCE RATIO	VOL. OF DIST.*	PER CENT OF BODY WEIGHT
1	13.6	10,200	16	35	172	–	23.5	–	5.9	43.4
			31	326	105	19.8	27.9	.70	9.4	69.1
			46	767	96	33.0	48.1	.69	9.8	72.1
			60	1,201	89	37.3	49.5	.75	10.1	74.3
			74	1,655	84	41.5	55.3	.75	10.2	75.0
			89	1,854	76	18.7	40.6	.43	11.0	80.9
			104	2,286	71	43.8	44.3	.99	11.1	81.6
			119	2,521	65	26.1	42.3	.62	11.8	86.8
5	8.2	4,060	15	188	94	14.4	17.5	.82	4.1	50.0
			26	368	84	21.0	28.9	.73	4.4	53.7
			45	629	72	20.4	33.9	.60	4.8	58.5
			60	856	63	25.6	32.6	.79	5.1	62.2
			75	1,030	55	22.7	32.6	.70	5.5	67.1
			90	1,170	48	20.7	36.4	.57	6.0	73.2
			105	1,304	42	22.8	32.8	.70	6.6	80.5
			120	1,449	37	28.5	33.7	.85	7.1	86.6
4	10.9	7,500	14	254	99	19.7	40.8	.48	7.3	67.0
		(53% excreted)	29	636	90	30.2	44.8	.67	7.6	69.7
			43	997	84	32.8	46.9	.70	7.7	70.6
			59	1,291	76	25.9	44.2	.59	8.2	75.2
			74	1,619	70	33.5	41.5	.81	8.4	77.1
			89	1,873	65	28.2	46.5	.61	8.7	79.8
			104	2,175	59	38.7	50.4	.77	9.0	82.6
			117	2,371	55	29.4	51.4	.57	9.3	85.3
3	8.2	2,050	15	147	40	26.5	18.8	1.41	4.8	58.5
		(45% excreted)	29	343	38	40.0	27.0	1.48	4.5	54.9
			44	467	34	25.9	34.5	.75	4.7	57.3
			59	557	32	20.0	30.8	.65	4.7	57.3
5	8.2	2,055	15	124	48	18.4	26.3	.70	4.0	48.8
		(69% excreted)	30	240	41	20.5	32.4	.63	4.4	53.7
			45	380	35	28.2	33.2	.85	4.8	58.5
			60	510	30	31.1	32.1	.97	5.2	63.4

*Volume of distribution (liters) $= \frac{a - b}{10\ c}$. a, Amount of drug injected (mg.); b, amount of drug recovered cumulatively in urine; c, plasma concentration in mg. %/0.93.

Under these conditions an adequate urine flow and a reasonably constant plasma level of creatinine were maintained. In the first three experiments, eight urine samples were taken by catheter at measured intervals of approximately fifteen minutes each, and five samples of blood were collected during the test period. In the last two experiments, four samples of urine and three samples of blood were collected. The plasma concentrations were plotted against time on semilogarithmic paper, and appropriate plasma concentrations were interpolated from the best straight line in accordance with the procedure described by Newman, Gilman, and Philips.[5] The data were used to calculate clearance ratios and volumes of distribution as shown in Table IV.

It is evident from the clearance ratios that Mandelamine undergoes reabsorption in the renal tubules. Closer scrutiny of the clearance data reveals two ratios which were manifestly out of line. These presumably resulted from analytic errors. It may be noted that the first Mandelamine and creatinine clearances were low. No explanation is offered for this observation but it is suggested that the depression in these clearance values may be the result of a temporary hypotension induced by the intravenous administration of the large doses of Mandelamine. No correction is included for possible binding of Mandelamine to plasma protein because the distribution of Mandelamine in whole blood indicates that the drug is freely diffusible. The volumes of distribution are consistent with the view that the drug is freely diffusible and rapidly attains equilibrium in its distribution in the compartments of body water.

The volumes of distribution observed within the first hour approximated the total volume of body water. With increasing time, however, the volumes of distribution increased to values as high as 87 per cent of the body weight. Because only 45 to 70 per cent of the total dose administered can be recovered in the urine, it is reasonable to assume that the apparent increase in the volume of distribution with time results from metabolism of the drug.

Acute Toxicity of Mandelamine.—Jenkins, Jack, and Drake,[6] Kolloff and Nelson,[7] and others have reported on the lack of toxicity of Mandelamine. In order to confirm and extend these findings, the acute toxicity of Mandelamine was determined in mice (weight, 17 to 22 grams; male; Carworth Farms (C.F.) No. 1 strain), guinea pigs (weight, 300 to 400 grams; male), rats (weight, 100 to 200 grams C.F.; male), and albino rabbits (weight, 3 to 4 kilograms; male). Buffered solutions of Mandelamine were injected intravenously. In all species, signs of acute toxicity consisted of convulsions, extension of the extremities, respiratory paralysis, and cardiac arrest. The data based on ten to twenty animals per dose point are summarized in Table V. The notable lack of toxicity of Mandelamine is in agreement with work reported earlier.

TABLE V. INTRAVENOUS TOXICITY OF MANDELAMINE

SPECIES	LD_{50} (GM./KG.)	LIMITS OF ERROR (%)
Mouse	3.75	97-104
Guinea pig	1.50	89-112
Rat	>4.0	-
Rabbit	>2.0	-

TABLE VI. CONCENTRATIONS OF MANDELAMINE IN HUMAN URINE

ORAL DOSE	SUBJECT	MANDELAMINE RECOVERED (MG. %) TIME IN HOURS														
		2	3	4	5	6	7	8	9	10	12	15	16	18	20	24
1 Gm.	1	5.0	34.6	77.7	142	98.3	83.3	73.6								
	(% excreted)	2.8	14.9	12.4	5.5	4.3	3.1	2.7								
	2	4.4	53.7	57.4	75.5	75.5	52.8	51.8		33.3	53.5					
	(% excreted)	0.6	3.9	6.9	6.2	4.9	6.0	8.3		5.2	6.4					
	3	12.8	65.8	69.2	114	79.4	123	82.3		33.5	50.3		14.9			
	4	4.0	17.7	34.4	-	107	95.8	70.7		-	46.9		26.1			
	5	8.2	19.1	29	103	91	89.1	72.6		59.5	27.8		29			
1 Gm. repeated after 6 hr.	6		22.5			194			190		153	72.6				
	7		10.6			91.9			246		170	112				
	8		5.1			70.6			34.6		106	80.3				
	9		74.5			85.2			65.8		92	74.5				
2 Gm. repeated after 12 hr.		5.7		54.2		159		310			217	325		434	248	-
	11	7.3		217		248		157		118	65	287		207	147	96

Buffered solutions of Mandelamine (1.25 to 10 per cent in physiologic saline) were injected into the shaved abdominal skin of the rabbit. An irritant drug, included to insure a positive response, produced severe necrosis at the sites of injection, but the buffer, saline, and Mandelamine solutions gave uniformly negative results.

Urinary Excretion of Mandelamine in Human Subjects.—Clinically, 1 Gm. doses of Mandelamine are administered by mouth three or four times daily. One gram of Mandelamine, therefore, was administered orally to each of five male volunteers,* with the results shown in Table VI. It may be seen that effective concentrations [1, 2] of the drug were attained in the urine within three or four hours, indicating a relatively slow absorption, and these concentrations persisted for at least another seven to eight hours. In a second experiment involving four normal male subjects, 1 Gm. of the drug was given and after six hours a second dose was administered. Urine samples collected at the times indicated in Table VI were analyzed for their Mandelamine concentrations with the results shown. In a third experiment (undertaken because of the notable lack of toxicity of Mandelamine) twice the recommended dose, namely 2 Gm., was administered to two volunteers and the dose was repeated in twelve hours. As shown in Table VI, this dose schedule also gave adequate urinary concentrations around the clock. It may be concluded from these data that 1 Gm. of Mandelamine administered by mouth three or four times daily will, in the presence of normal renal function, produce and maintain antibacterial concentrations of Mandelamine in the urine.

TABLE VII. URINE CONCENTRATIONS AFTER 1 GM. MANDELAMINE THREE TIMES DAILY FOR FOUR DAYS

SUBJECT	DAYS				MEAN VALUES
	1	2	3	4	
J. M.	130.7	101.6	68.8	110.4	102.9
J. F. R.	64.8	71.7	63.4	50.8	62.7
R. E.	93.9	52.2	73.6	75.5	73.8
M. R.	32.7	41.6	58.1	55.2	46.9
Z.	63.4	73.6	93.9	66.8	74.4
T.	127.8	106.7	77.4	101.6	103.4
G.	68.8	113.3	90.0	85.1	89.3
L.	126.9	108.5	104.5	126.9	116.7

In order to establish more convincingly the effectiveness of the accepted dosage, nine volunteers were given 1 Gm. of Mandelamine by mouth at 9:00 A.M., 5:00 P.M., and before retiring. These experiments were continued for a period of four consecutive days. It should be emphasized that these subjects continued in their normal occupations with no limitations imposed upon the fluid or food intake. Daily urine samples, collected at 10:00 A.M., were analyzed with the results shown in Table VII. All urinary concentrations were within or above the range of antibacterial effectiveness. The mean of all values was $83.3 \pm .308$. Although the figures varied significantly among themselves, it could be shown by analysis of variance that differences within individuals were not significant within the observation period of four days. It may be concluded,

*Commercial Mandelamine tablets were used in all human experiments.

therefore, that a dose of 1 Gm. three times daily will, in the presence of normal renal function, produce antibacterial concentrations of Mandelamine in the urine.

Carroll and Allen[8] have reported that, following ingestion of Mandelamine, the urine of 96 per cent of their patients became and remained acidic without other medication or restriction of diet or fluid intake. Our findings in normal subjects are in agreement with those of Carroll and Allen. Invariably the urinary pH was lowered, and in all instances a single dose of 1 Gm. was sufficient to lower the urinary pH to about 6. The urinary pH was maintained between 5.8 and 6.4 when 1 Gm. of the drug was administered three times daily for four days.

SUMMARY

Mandelamine readily penetrates the red blood cell and distributes itself in proportion to the water content of whole blood. In the dog the volume of distribution approximates the total volume of body water. The volume of distribution increases with time, presumably because of extrarenal activity. The drug undergoes tubular reabsorption in the dog. These observations are consonant with the view that the drug persists in the organism. In human subjects, 1 Gm. of Mandelamine administered three or four times daily is adequate to produce and maintain antibacterial concentrations of the drug in the urine. The notable lack of toxicity of Mandelamine is confirmed.

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LABORATORY METHODS

IMBEDDING OF PATHOLOGIC SPECIMENS IN TRANSPARENT PLASTIC

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INTRODUCTION

IN THE teaching of biologic sciences it is essential to be able to demonstrate specimens of animals and soft tissues. This is even more true of some of the medical sciences, particularly gross anatomy and pathology. The preservation of such specimens always has presented a very difficult problem. The most usual and probably the most satisfactory method at present in general use consist in fixing the organs or slices of organs in various fluids, most of which as a rule contain formalin, with the addition of various substances designed to retard as much as possible the loss of color and shrinkage. These specimens generally are mounted in round or square glass jars. Even with the greatest care, however, and with the best-known procedures, this method offers numerous disadvantages. First of all, color preservation is good to fair for only a relatively short period of time, because on standing there is progressive bleaching. In the second place, shrinkage takes place with distortion of the specimen. Third, there are the factors of weight and difficulty in handling bulky museum jars, with the danger of breakage, difficulty of transportation, and so on. In addition there is generally great difficulty in perfectly sealing such jars, with the necessity of adding or renewing the fluid from time to time, and finally, the preserving fluid becomes discolored and after a period of time needs renewing. Very often, also, turbidity occurs.

Employing substances generally known as plastics which can be transformed from the liquid to the solid state, successful attempts have been made, particularly by Sando,[1] to imbed minerals, insects, and similar structures in transparent blocks.* However, the procedure involved the use of chemical dehydrating agents and it could not be applied successfully to animals or organs of animals containing large amounts of water without considerable loss of form and color. The combination of drying from the frozen state (by sublimation of water vapor), impregnation of the dried tissue with the liquid monomer, polymerization, and subsequent imbedding in a transparent solid plastic has yielded good preservation of both color and form of the most delicate human, animal, and vegetable tissues.

The use of dehydration by sublimation of water from the frozen state was suggested by the fact that plasma and other complex colloidal substances can be dried without deterioration.[3]

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*Very recently Kampmeir and Haviland[2] have published a method for the mounting of wet anatomic preparations in Castolite. This method does not preserve the natural color in specimens.

Thus far the work has been directed toward developing a satisfactory basic technique. Since the time of the publication of a preliminary paper[4] the method has been considerably improved and applied to imbedding of practically every organ of the human body and numerous other animal and vegetable specimens. The early promises of this method have been at least partly realized. The present publication deals mostly with presentation of the technique which has given the most constant and satisfactory results. This technique suggests itself for application for the collection of anatomic and pathologic specimens for teaching in schools, medical and veterinary, and for the teaching of biology, zoology, botany, and the allied sciences.

PROCEDURE

Material.—The material used for imbedding is uninhibited monomer consisting of a mixture of 90 per cent methyl methacrylate and 10 per cent ethyl methacrylate. This mixture is generally packed in dry ice to avoid polymerization prior to use.*

The uninhibited liquid monomer must be maintained in the refrigerator to avoid premature polymerization. Under these conditions the liquid monomer can be maintained for several months.

Preparation of Specimens.—The technique described below is intended especially for slices of organs.

The fresh specimen should be trimmed carefully and freed as much as possible of excessive fat and loose blood or fluid exudates. Slices of organs need not be limited in width to any size but they should be reasonably flat and should not exceed 2 cm. in thickness. Organs or slices of organs which have a tough capsule should be freed of it if possible. This is particularly true of the kidney.

Freezing: Freezing should be accomplished in a relatively short period of time, but instantaneous freezing is by no means necessary or even desirable. It is important not to allow the specimen to remain exposed to the dry atmosphere of low temperature cabinets for too long a period of time, in order to avoid superficial drying which would cause loss of color and distortion in the finished specimen. The best technique for slices of organs is as follows: Into a pan of proper depth, preferably metallic (although glass will do almost as well), pour water to a depth of about 1 cm. and allow it to freeze and cool to a temperature of about –20° C. During this process a raised lump often forms in the middle. This should be carefully scraped off in order to have a perfectly flat surface. Immerse the specimen in water and while it is thoroughly wet lay it carefully on the formed base of ice. Allow it to freeze, so that it becomes firmly attached to the base of ice; then add in rapid succession thin layers of water (0.5 to 1 cm.). Each layer of water is allowed to freeze before addition of a new one. Continue the procedure until the whole specimen is covered with approximately 1 cm. of ice (Fig. 1). The block of ice is then removed from the form and trimmed so that there is no excessive ice on the sides. For whole organs, proceed as follows.

*This material is commercially known as Plexiglas. It can be obtained from the Rohm and Haas Company, Philadelphia, Pa.

Prepare a base of ice as outlined. Place the organ, thoroughly wetted, on the base and allow it to freeze. When the specimen is thoroughly frozen and fixed to the base, with an atomizer spray it several times with a thin layer of ice. A layer of 1 or 2 mm. in thickness is enough to protect the surface of the organ from drying and subsequent loss of color and shrinkage. The specimen is then completely imbedded in ice by addition of successive layers of water of about 1 cm. in thickness. Carefully avoid pouring the water on the organ itself in order not to melt the glaze of ice.

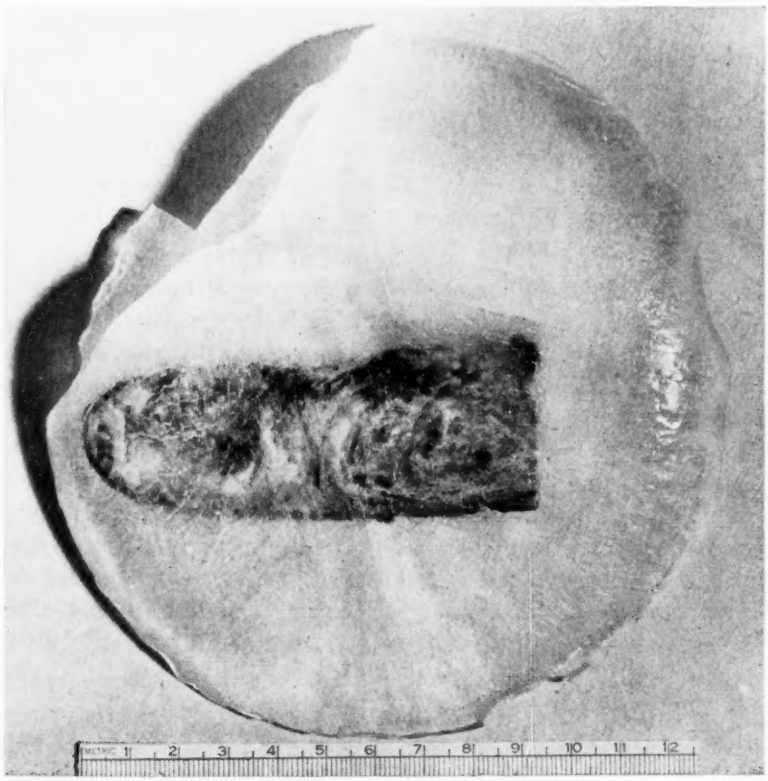

Fig. 1.—The fresh specimen frozen and encased in ice.

Before placing the frozen block in the drying apparatus, it is desirable to chip off as much as possible the excess of ice in order to reduce the period of drying. Specimens may be preserved in the frozen state without appreciable deterioration for more than a year. This operation is not essential except as a timesaver. The ice blocks must be thoroughly chilled to –20° C. or less before they are transferred to the drying apparatus.

Drying of Specimens From the Frozen State: Any apparatus of proper capacity designed for the drying of biologicals from the frozen state can be used for this purpose. It must be capable of maintaining a temperature of –12 to –15° C. or less in the specimen itself while the water is being removed by

sublimation. An apparatus previously described[3] or any similar one has been found to yield excellent results. It is desirable to place the blocks of ice containing the specimens to be dried in a loosely tied bag made of a single layer of gauze (12 mesh). The wrapped specimens are placed in wire baskets or on supports of the same material and so arranged as to allow a free flow of the water-vapor. For medium sized specimens (approximately 5 to 7 cm. in overall thickness and of any width fitting the drying chamber) and with the apparatus mentioned, five days are sufficient for complete drying. The water jacket heating the drying chamber should be maintained at a temperature of 37 to 40° C. Higher temperatures may be injurious to specimens containing large quantities of fat. Very large organs such as an entire brain require about ten days for complete drying. Certain specimens, for example an entire animal or fetus, containing trapped air, such as that contained in the gastrointestinal tract, or soft tissues in a hard or bony shell at times show some distortion from shrinkage in the final specimen. This can be avoided by drilling holes before freezing. Otherwise drying from the frozen state, when properly carried out, causes no distortion of the specimen nor loss of color or structure.

Trimming and Preservation of Dried Specimen: Once the specimen is dried it should be carefully maintained so by placing it in a glass desiccator containing a suitable dehydrating agent, such as magnesium perchlorate (trihydrate). Specimens may be kept in the dried state before imbedding for several weeks or even months without appreciable deterioration. For optimal results, however, the dried specimen should be imbedded as soon as convenient. The specimen can be trimmed with a very sharp knife in order to remove all loose portions and for the purpose of improving the specimen. It has been noted that retrimming of a slice of organ after drying for the purpose of obtaining a very smooth and even surface allows a much better view of the intimate structure of the tissue. Excessive fat can be removed readily at this time, avoiding damage to the specimen. Dust and loose particles must be removed carefully from the surface of the organ with a soft brush.

Saturation With the Liquid Monomer: The dried specimens appear to have lost their color and texture. These are restored immediately upon immersion in the liquid monomer at room temperature. It is most essential to obtain a very thorough saturation of the specimen with the liquid monomer, which entirely replaces all spaces previously occupied by water. This is accomplished by submitting the jar containing the specimen to a high vacuum in a protected desiccator. As a result of the procedure of producing a vacuum and releasing it many times in succession, it will be noted that the specimen will sink in the medium and will issue no more bubbles. At this stage the specimen is ready for the passage to the thickened monomer, unless it is desirable to prolong the immersion in the monomer for the purpose of removing excessive amounts of pigment, a procedure which should be applied to such organs as the liver. With specimens of liver or organs containing other diffusible pigments or fat, the liquid monomer should be changed a number of times during the process of saturation, until it remains clear and practically colorless.

Preliminary Partial Polymerization of the Medium.—

Equipment and Reagents:

1,000 c.c. Erlenmeyer or Florence flask (Pyrex).

500 c.c. of the mixed uninhibited monomer.

100 mg. benzoyl peroxide.

Water bath, electrically heated and with spark-proof switch.

A chemical hood. If provided with a motor-driven aspirator the motor should be of the induction type (spark proof).

Procedure: To 500 c.c. of the monomer in the Erlenmeyer flask add 100 mg. of benzoyl peroxide. Immerse the flask so that the level of the manomer is at the level of the water of the water bath, maintained under a hood. Bring the water to the boiling point. The boiling point of the ethyl methacrylate is about 110° C., but the polymerization is an intensely exothermic reaction and the monomer will soon boil. Remove the flask from the water bath; the monomer will boil without additional heat. Allow boiling to proceed for two to three minutes. Carefully cool without unnecessary jarring but with a constant swirling motion under running cold water until bubbling ceases. Continue cooling for approximately ten minutes until a temperature of about 40 to 45° C. is reached. Place in the refrigerator (4° C.) until ready to use. The material can be thus stored for several weeks.

Since the vapors of methyl methacrylate are highly inflammable they should be kept from all open flames, sparking switches, motors, and so forth. If possible, place switches outside the hood. At all stages the prepolymerization process should be closely observed since the reaction is intensely exothermic and the monomer will solidify or boil over and form an opaque mass.

Amounts larger than 500 c.c. may be prepared. However, greater difficulty is encountered in cooling rapidly and therefore the monomer may go on to complete solidification.

The consistency of the final product is directly proportional to the length of time the monomer is allowed to boil. The times given here produce material which is about the consistency of extra heavy oil or molasses.

Imbedding of Specimens.—For the imbedding of specimens and the preparation of solid bases a temperature of 40 to 45° C. is generally employed. For this purpose any hot-air oven may be used provided the thermoregulator is spark proof. A thermoregulator of the sealed mercury type is satisfactory.

When large specimens are imbedded the heat of polymerization may be sufficient to produce a temperature considerably higher than 40 to 45° C., and if the excessive heat is not rapidly dissipated bubbling will occur and thus spoil the specimen. In these instances a proper temperature can be maintained by a blower, activated by a thermostat, set to close the motor circuit at 46° C. (Fig. 2).

It is desirable to use glass containers for the final imbedding. Metallic containers have not proved as satisfactory. Ordinary square refrigerator storage containers or baking dishes as well as glass photographic trays have been used with good results. It is preferable to prepare the receptacles with a hard base

of plastic on which to rest the specimen. This hard base is prepared by pouring into a clean dish of suitable size and depth a layer of about 2 cm. of the partly polymerized monomer. For this purpose very thick medium can be used. Cover the dish with two or three layers of cellophane, tightly fitted, and place in the refrigerator until the medium is absolutely free of air bubbles. Place at 45° C. until the medium is entirely hard. The base is now ready to receive the specimen. The following procedure is recommended.

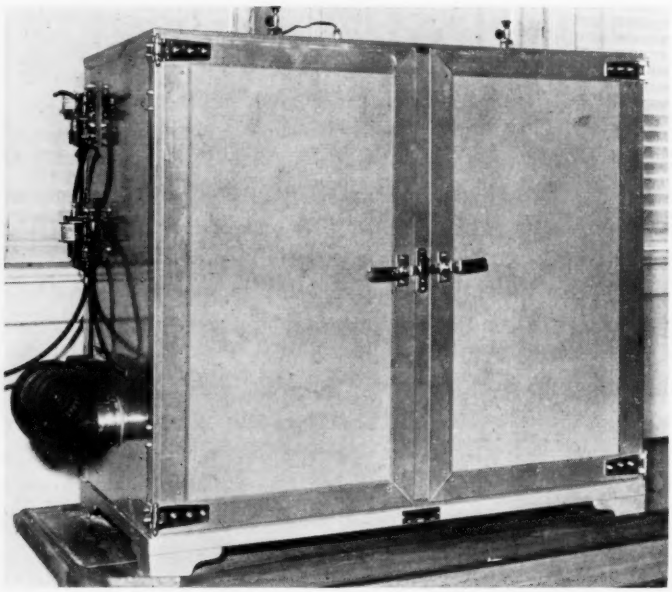

Fig. 2.—Oven with blower.

For flat slices of organs, or thin organs such as intestines, pour over the preformed hard base a small amount of the liquid monomer and with it thoroughly wet the base as well as the sides of the container. Pour the liquid off and drain rapidly. Pour over the hard base a layer of the thickened monomer calculated to be just enough to cover the specimen. If bubbles have formed, cover with cellophane and place in the refrigerator until they all have disappeared. Do not allow the poured thickened medium to stand for any period of time without a cover, particularly at room temperature or in the oven, because a tough film forms which renders subsequent operations difficult. When the medium is freed of air bubbles, remove the specimen from the monomeric medium and place it edgewise in the thickened medium by lowering it very gradually in such a manner as to avoid trapping air bubbles. It may be necessary at this time to add some of the thickened medium to cover the specimen completely, but under no condition add at this time a layer thicker than 2 to 3 centimeters. Cover the container with cellophane, and if air bubbles have been formed, replace in the refrigerator until clear. Then set in the oven at 45° C. for polymerization. A block 3 cm. thick will harden in approximately one week

at 45° C. Successive layers of 1 to 2 cm. in thickness may be added to obtain a block of suitable size.

Imbedding of Large Irregular Organs: The technique just described applies well to slices 1 to 2 cm. thick of any organs such as liver, spleen, lymph nodes, thyroid, lungs, kidneys, tumors, and to flattened out specimens from large vessels, intestine, gall bladder, and so on.

Large irregular organs, however, such as heart, lung, uterus, brain, and whole liver require considerably more time and care. It is generally necessary

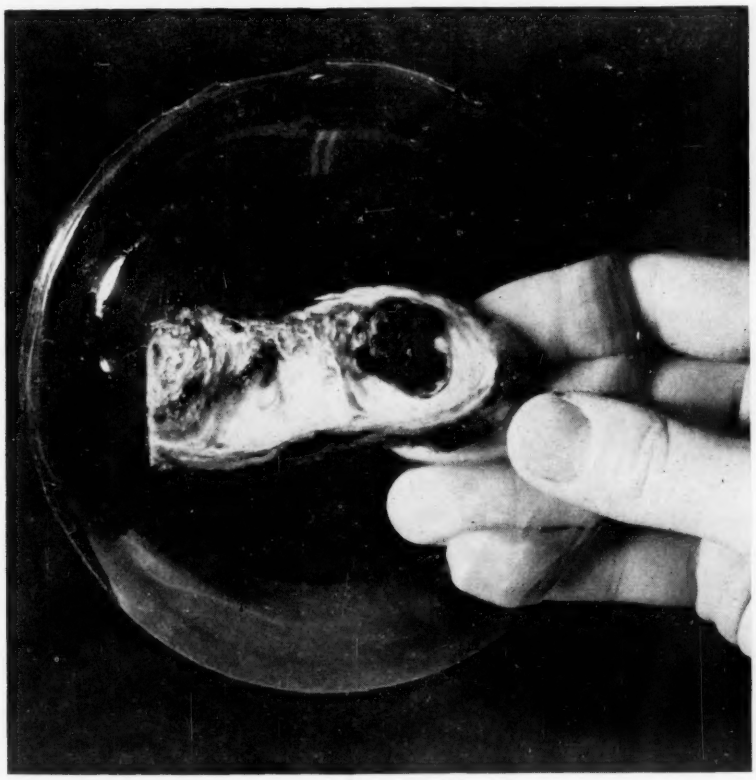

Fig. 3.—Specimen imbedded in the block of transparent plastic.

to begin the operation by preparing a base of suitable size. When this is obtained, the organ which is removed from the liquid monomer is coated with a thin layer of thickened monomer, by immerision, and placed on the base. The container with the base and the specimen is then very carefully sealed with many layers of cellophane or similar material. It is placed in the refrigerator so that it is thoroughly freed of bubbles and then placed in the oven at 45° C. This process is repeated until the organ is covered with a substantial layer. Great care must be exercised to cover the whole organ and to avoid trapping air bubbles. When the organ is thus glazed and fixed to the base, proceed carefully to imbed it by adding successive layers not exceeding .5 cm. in thickness in order

not to soften excessively the glazing on the organ. Whereas the imbedding of thin slices of organs offers no difficulty, the imbedding of entire large specimens challenges the skill and ingenuity of the operator. The results however are well worth the effort.

Finishing of the Specimen: When the specimen is completely solidified, the edges of the plastic are detached from the glass with a sharp knife. On cooling, the blocks detach themselves from the walls of the container and will easily fall out with gentle tapping. (Fig. 3.) The finishing of the block is carried out as follows. The solid block first is rough-cut to the approximate size and shape of the final block desired with an ordinary band, circular, or jig saw. If a lathe is available it is well to turn the faces of the block down, since this will insure an absolutely flat surface. Since Plexiglas has machining qualities similar to those of brass and copper, metal cutting tools of this type can be used. A coolant (water, or soap and water) may be used if desired. If no lathe is available the block may be faced to a smooth (not accurately flat) surface by fastening emery paper to a block of wood and hand sanding with a circular motion. It is advisable to start the sanding with a coarse grade of emery paper and finish with a fine grade. If a sander is available the work may be accomplished much more quickly, but the final sanding should be a wet one, which will give a soft satin finish that can be buffed easily. Best buffing results are obtained with a very soft, open type of buffing wheel and an abrasive which is a combination of fine alumina with wax or grease binder, and a polishing tallow. The block, when finished, should have a high luster and be free from color and as transparent as the finest optical glass.

Reimbedding After Cutting of Imbedded Specimens: To obtain a perfectly smooth surface of slices of organs or tumors, the imbedded specimen can be cut through with a fine band saw or similar tool and then polished as outlined to the point of a smooth, even, perfectly flat surface, or most of the clear block can be cut away and the specimen made flat by dry grinding. The specimen is then reimbedded as follows: Place the entire block with the exposed tissue surface uppermost in liquid monomer, and by means of high vacuum resaturate the tissue. Place face up in a suitable container and proceed to reimbed, following exactly the technique outlined previously. To surely avoid formation of bubbles on the raw surface of the exposed organ, it is desirable to store the specimen overnight in the icebox prior to polymerizing at 45° C.

Very thin, practically transparent sections can be obtained by proceeding as outlined and then cutting, polishing, and imbedding the other surface of the slice. These specimens are particularly valuable for teaching.

Removing Trapped Air Bubbles: Trapped air bubbles can be removed from an otherwise satisfactory block by cutting and/or drilling with an instrument activated by a high-speed motor; 5,000 revolutions per minute are satisfactory for this purpose. A tool with a flexible shaft is most desirable. Several types of drills may be obtained for practically any purpose.

Fig. 4.—*A*, Fresh specimen from xanthoma of the knee; *B*, specimen after drying and trimming; *C*, specimen imbedded in finished block of Plexiglas.

Commonest Failures in Imbedding of Specimens in Plexiglas.—These can be listed as follows.

(1) Loss of color. Cause: Previous fixation in formalin or prolonged immersion in liquid monomer.

(2) Shrunken portions. Cause: Incomplete immersion in water during freezing.

(3) Turbidity of the medium. Cause: Improper or incomplete drying, or excess of fat in the specimen.

(4) Formation of bubbles. Cause: Excessive heat during polymerization.

(5) Diffusion of pigment from the specimen. Cause: Too slow polymerization. A common occurrence with certain specimens, such as liver.

(6) Disintegration of the specimen. Cause: Generally too prolonged exposure to monomer at high temperature.

(7) Fuzzing of the specimen at the periphery. Cause: Unknown. Specimens thus affected may be cut so as to expose the inner portion and reimbedded with good results.

(8) Formation of white precipitates. Cause: Unknown. A not uncommon occurrence in imbedding very large specimens such as hearts and lungs. Avoid prolonged immersion in partially polymerized methyl methacrylate by adding thin successive layers.

(9) Brittleness and loss of structure in cutting. Cause: Improper impregnation of dried specimen with liquid monomer.

DISCUSSION

With the process described one can obtain preservation of the color and form of human specimens and similar material not possible with any other method known to us. These points are illustrated in Figs. 4 and 5. Fig. 4, *A* shows a fresh slice through a portion of a large xanthoma of the knee, with areas of hemorrhagic degeneration. Fig. 4, *B* is the same specimen after drying and trimming. Fig. 4, *C* shows the specimen imbedded in the finished block. Figs. 1 and 3 show the same specimen frozen in a block of ice and at the stage of imbedding in the rough block of Plexiglas. Fig. 5 shows the tumor preserved in a block of Plexiglas and a similar portion preserved in Klotz solution for a period of one year. Fading is already very noticeable in the specimen preserved in fluid.

For a slice of any organ up to 1 or 2 cm. in thickness, it is approximately three weeks from the time the specimen is frozen to the time the finished block is obtained. The technique of cutting imbedded specimens, polishing the cut surface, and reimbedding is recommended for optimal results. This process causes thorough impregnation of a perfectly smooth section of the specimen, particularly in such organs as liver, kidney, lung, and so on, thus permitting observations of minute details of the structure.

The technique for the imbedding of sections of organs, of tumors, or of entire animals can be said to be fairly well standardized and to produce fairly uniform and satisfactory results. Less constant results are obtained in im-

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Fig. 5.—Left, portion of a xanthoma of the knee, preserved in Klotz solution for a period of one year.

Right, similar portion of the same tumor preserved in a block of Plexiglas.

bedding entire large organs, such as an adult heart, lung, or brain. With this type of material the results have been at times brilliant but not uniformly so, and failures have been just as common as successes. It is expected that improvement in the technique of imbedding and possibly in the preparation of the monomer employed may greatly aid the solution of the problem. With proper illumination, transparent blocks of Plexiglas containing organs or portions of organs have a particularly lifelike aspect. These specimens can be handled readily without danger of breakage, and they remain absolutely unchanged for periods in excess of at least five years.

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A CONVENIENT AND RAPID PROCEDURE FOR TOTAL CHOLESTEROL ESTIMATION USING AN ACID CHLOROFORM EXTRACTION

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THIS paper describes a simple rapid method for the determination of total cholesterol in blood serum.

The alcohol-ether extraction methods are time consuming because they require transfer and evaporation of the solvent.[1-4] Recently methods have been reported for the determination of total cholesterol in one test tube by the addition of acetic anhydride directly to the blood serum in the presence of sodium sulfate[5] or of dioxane.[6] These methods require procedures which need several hours for completion.

The method described herein will yield the desired result in approximately forty-five minutes. It lends itself to use in the routine laboratory in which large numbers of determinations must be done simultaneously. For example, thirty total cholesterol determinations in duplicate may be completed in two hours.

The present procedure depends upon the fact that dilute acids or alkalies, or saturated solutions of sodium sulfate, will split cholesterol from proteins in the presence of chloroform. Simultaneously, when this extraction is carried out in a shaking machine, the chloroform completely extracts the cholesterol.

The preferred procedure, therefore, consists of placing the serum in a test tube, adding dilute sulfuric acid and chloroform, stoppering the tube with a fat-free rubber stopper, and centrifuging. The precipitated protein collects at the interface of the acid and the chloroform. The supernatant liquid and the protein button may be aspirated off with the aid of water suction and an aliquot of the chloroform layer taken. Acetic anhydride is added and the color is developed at constant time and temperature in the dark by the addition of concentrated sulfuric acid. This color may now be read in a photoelectric colorimeter.

When this extraction procedure is used, maximum color development occurs in seven minutes at 25 to 26° C. The color is therefore developed in a constant temperature bath at 25 to 26° C. and read after seven minutes.

Fig. 1 is a straight curve obtained by plotting concentration against the reading in a Klett-Summerson photoelectric colorimeter with a No. 60 filter. A similar curve is obtained using 625 mμ as the absorption beam with the Coleman spectrophotometer.

A box was designed to shake simultaneously 150 to 200 tubes for cholesterol extraction. This box is mounted on a standard large-sized Kahn reciprocating shaker. Fig. 2 shows this box, made of five-ply wood, the partitions, and the rubber-faced plungers. This shaking box is also suitable for holding four bottles in place and shaking them simultaneously.

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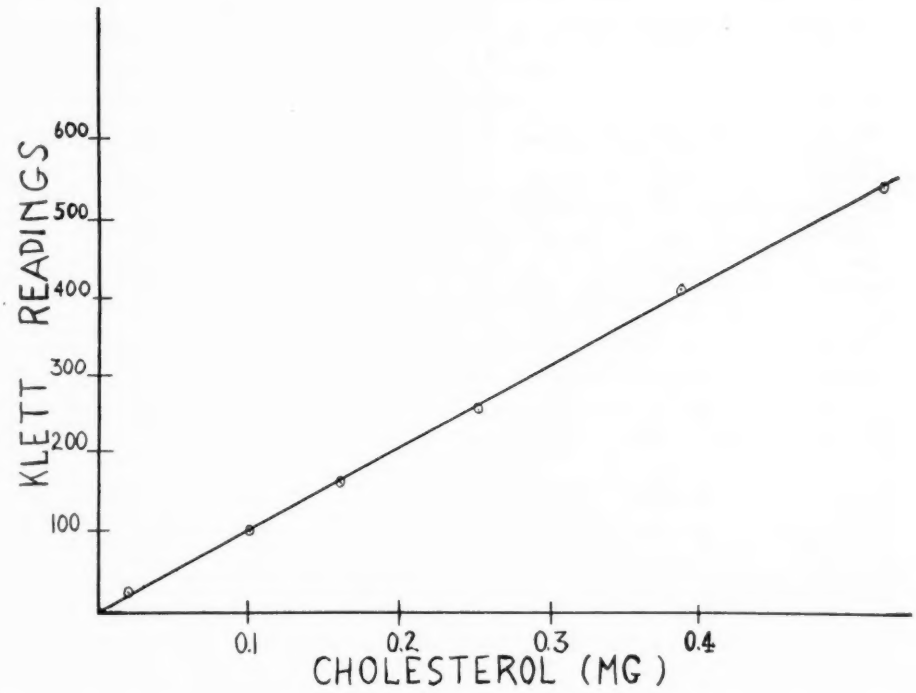

Fig. 1.—Standard curve plotting milligrams of cholesterol against Klett readings. Total volume, 3.1 milliliters.

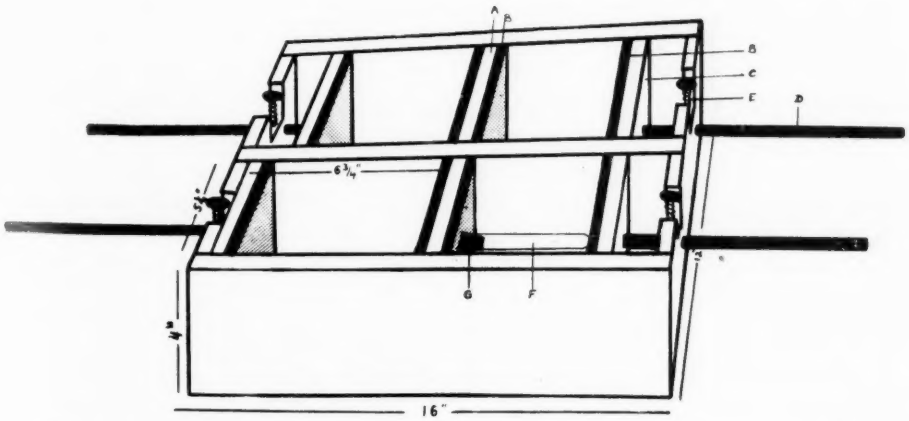

Fig. 2.—Container with four compartments for shaking 150 to 200 stoppered test tubes. *A*, Wooden partition; *B*, rubber facing; *C*, movable wooden partition; *D*, metal rod; *E*, clamping screw; *F*, test tube; *G*, rubber stopper held in place.

By moving the plungers forward and fixing them in place with set screws, the rubber-stoppered test tubes are held tightly in place as they are shaken back and forth.

REAGENTS

10 per cent sulfuric acid (10 Gm. of concentrated H_2SO_4 made up to 100 ml.).

Chloroform, analytical grade.

Acetic anhydride, analytical grade.

Cholesterol standard, 0.2 mg. per millimeter. Forty milligrams of cholesterol recrystallized from acetone and dried are dissolved in less than 200 ml. of acetic anhydride in a volumetric flask at 60° C.; when completely dissolved, the solution is made up accurately to the 200 ml. mark.

Recovery solution, 0.025 mg. per millimeter. Fifty milligrams of cholesterol are dissolved in 200 ml. of chloroform; 10 ml. of this solution are diluted to 100 ml.

Fat-free stoppers No. 0 are allowed to soak in chloroform for about half an hour and then dried with gauze. This is repeated each time before use.

PROCEDURE

To a 15 ml. test tube is added 0.1 ml. of blood serum; to this is added 4 ml. of 10 per cent sulfuric acid. The acid should be blown from the pipette with force in order to preprevent the protein from adhering to the walls of the tube. To this are added exactly 4 ml. of chloroform from a burette. For the recovery study, 4 ml. of the recovery solution are added instead of the 4 ml. of chloroform. The tube is then stoppered tightly and shaken in a shaking machine for twenty to thirty minutes. It is then centrifuged. The supernatant acid and the protein at the interface are then aspirated off with the aid of water suction. A 2 ml. aliquot is now taken from the remaining chloroform, to which is added 1 ml. of acetic anhydride. This is done by touching the tip of the pipette lightly to silicone grease, inserting the pipette into the chloroform, blowing off the silicone grease, and aspirating the chloroform with a water aspirator. The solution is mixed and 2 drops (0.04 ml.) of concentrated sulfuric acid are added and the mixture is shaken vigorously. The color is allowed to develop for seven minutes in a water bath at 25 to 26° C., in the dark, and then read on a Klett-Summerson photoelectric colorimeter with a No. 60 filter, or on the Coleman spectrophotometer using 625 mμ as the absorption beam.

STANDARD AND BLANK

The standard is developed and read at the same time as the unknown. It consists of 1 ml. of the 0.2 mg. per millimeter cholesterol standard, plus 2 ml. of chloroform and 0.04 ml. of concentrated sulfuric acid. It is developed and read in the same manner as the unknown.

The blank consists of 1 ml. of acetic anhydride, 2 ml. of chloroform, and 0.04 ml. of concentrated sulfuric acid.

CALCULATIONS

The concentration of the cholesterol in the unknown may be read off the standard curve. Since this curve is a straight line the formula below will yield the cholesterol values in milligrams per cent when 0.1 ml. of serum is used.

$$\frac{\text{Density of unknown}}{\text{Density of standard}} \times 0.2 \times \frac{100}{0.05} = \text{mg. \% of cholesterol}$$

RESULTS

The results obtained are listed in Table I. It is apparent that the results compare favorably with those obtained by extraction with Bloor's reagent. Table II indicates the amounts of cholosterol recovered when known amounts are added, as indicated under "Procedure."

TABLE I. COMPARISON BETWEEN THE KAYE METHOD AND THE AUTHORS' METHOD
(The values are expressed as milligrams per 100 c.c. of serum. Each value is the average of duplicates.)

SAMPLE	NEW METHOD	KAYE METHOD	SAMPLE	NEW METHOD	KAYE METHOD
1	228	219	6	184	182
2	300	289	7	268	269
3	189	189	8	139	139
4	309	307	9	273	276
5	202	195	10	265	262

TABLE II

NUMBER OF DETERMINATIONS	CHOLESTEROL ADDED*	MEAN AMOUNT	AVERAGE DEVIATION FROM MEAN
18	0.1 mg.	0.098	±0.002

*See Procedure for details.

SUMMARY

A convenient method is described for determining total cholesterol in sera employing a chloroform-acid extraction.

The results are available within forty-five minutes after the beginning of the determination.

The results are comparable to those obtained with Bloor's method of extraction.

This procedure is recommended where large numbers of determinations are required in a short time.

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METHODS FOR THE CHEMICAL DETERMINATION OF CORTICOSTEROIDS IN URINE AND PLASMA

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THE methods here described derive from one suggested in abstract[1] but found inadequate. The underlying principles are extraction and partial separation of neutral steroids from urine or plasma and oxidation of the α-ketol or α-glycol side chains of the C_{21} steroids of adrenal cortical origin with periodic acid[2] followed by colorimetric measurement of the formaldehyde formed in the oxidation.[3] Oxidation and colorimetry proceed in the manner described for determination of mannitol[4] with the exception that formaldehyde is distilled from the reagent mixture before treatment with the chromogenic reagent. This modification has been described independently[5] for determination of urinary cortin in a procedure which corresponds generally with the one here described.

The greater specificity of a procedure which depends on periodic oxidation as compared with sugarlike reduction of copper reagents is now accepted by many workers in the field. It is therefore desirable to present together analogous methods for estimation of corticosteroid in both urine and plasma which include this principle.

APPARATUS

All-glass, standard taper flasks, distillation apparatus and semimicro flasks and condenser.

Tall (200 × 20 mm.) glass test tubes calibrated at 10 or 12.5 c.c.; Lewis and Benedict blood sugar tubes are suitable.

Spectrophotometer* or other suitable photocolorimetric apparatus.

Automatic pipette (5 c.c.) for rapid, free delivery of chromotropic acid reagent.

REAGENTS

Solvents. (1) Chloroform, chemically pure, redistilled. (2) Glacial acetic acid, Baker's special (empyreuma-free). (3) Alcohol-ether: 3 volumes 95 per cent alcohol to 1 volume of ethyl ether. (4) Petroleum ether, boiling point, 30 to 60° C. (5) Acetone, chemically pure.

Sodium sulfate, anhydrous.

Sodium hydroxide, 0.1N.

Saturated aqueous solution of magnesium chloride.

Periodic acid reagent. Potassium periodate, 0.03M in 0.25M sulfuric acid.

Stannous chloride reagent. Prepared daily by dissolving about 1.4 Gm. stannous chloride in 50 c.c. of 2.5N HCl. The solution is titrated to a starch end point with periodic acid reagent and adjusted so that 10.2 c.c. of periodic acid reagent oxidizes 10 c.c. of stannous chloride reagent.

Chromotropic acid reagent. Dissolve 0.2 Gm. of chromotropic acid (1,8-dihydroxynaphthalene sulfonic acid) in 4 c.c. of water in a 100 ml. volumetric flask and make up to volume with 15M sulfuric acid. The solution is prepared daily.

Approximately 9M sulfuric acid.

From the Research Division of the Cleveland Clinic Foundation.
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*Coleman Junior Clinical Spectrophotometer (Model 6A).

PROCEDURE

Urinary Corticosteroid.—

Collection and Extraction: A twenty-four- or twelve-hour night urine sample is collected. Depending on the adequacy of refrigeration and the speed of beginning extraction, no preservative need be added or there may be added 5 c.c. of chloroform or 1 c.c. of 1 per cent aqueous Merthiolate (Lilly).

A four- or six-hour aliquot of the sample is brought to about pH 1.0 with concentrated HCl; 100 c.c. of chloroform are added. The mixture is shaken once and refrigerated until analyzed.

The urine is extracted four times with 100 c.c. portions of chloroform by shaking for fifteen minutes with each portion. The urine layer is discarded from a separatory funnel and the combined extracts and emulsions are centrifuged. The remaining urine layer is discarded; the extracts and emulsions are combined and dried by addition of sodium sulfate. The clear supernatant extract is filtered through glass wool.

This extract is chilled, washed twice with 0.1 volume of cold 0.1N NaOH, and once with water. Each washing is back-extracted with an equal volume of chloroform. These extracts are added to the washed chloroform extract and the NaOH and water are discarded.

The washed chloroform extract is evaporated in vacuo at less than 50° C. in an all-glass distillation apparatus to a volume of about 10 cubic centimeters. At this point the extract is divided in two equal portions and each is quantitatively transferred by rinsing with small volumes of chloroform into round-bottomed distillation flasks of 25 c.c. capacity. Evaporation is continued to dryness.

Colorimetric Assay: (1) Oxidized sample: The dried residue from one of the two flasks is dissolved with 0.5 c.c. of glacial acetic acid. When the flask residue is thoroughly wetted, 8.5 c.c. of water are added and to the mixture 0.5 c.c. of periodic acid reagent is added. The mixture is allowed to stand at room temperature for thirty minutes, when oxidation is arrested by addition of 0.5 c.c. of stannous chloride reagent. (2) Unoxidized sample: The residue is brought into solution as described and made up with water to 9 cubic centimeters. Then 0.5 c.c. of stannous chloride reagent is added, followed immediately by 0.5 c.c. of periodic acid reagent. (3) Blank on reagents: Oxidized and unoxidized reagent blanks are prepared from volumes of all reagents equal to those used in analysis.

The distillation flask is attached to a semimicro condenser. The elongated outlet tube of the condenser is placed under the meniscus of 1.0 c.c. of water in a 10 c.c. volumetric flask. About 8 c.c. of distillate are collected by careful heating over a microburner. The distillate is then made up to volume with water.

Three cubic centimeters of distillate are placed in a tall glass test tube and 5 c.c. of chromotropic acid reagent are rapidly mixed in. The tube is placed in a boiling water bath for thirty minutes. At the end of this time it is rapidly cooled to 25° C., made up to volume (10 or 12.5 c.c.) with 9M sulfuric acid, and stabilized to 25° C. in a water bath. Color density (D) is measured at 570 microns.

Calculation: The color density due to liberation of formaldehyde from the unoxidized sample is subtracted from the color density of the oxidized sample. From this is subtracted the corresponding color density obtained from the difference between oxidized and unoxidized blanks. The resultant color density represents formaldehyde liberated from corticosteroid-like substances in the extract of urine. The amount present is found by reference to a calibration curve prepared from oxidation and colorimetry of desoxycorticosterone in 20 per cent alcohol. The result is expressed as milligrams corticosteroid per twenty-four hours.

Plasma Corticosteroid.—

Collection and Extraction: The plasma from about 50 c.c. of fresh, heparinized blood is added drop by drop with stirring to 5 volumes of 3:1 alcohol-ether. The protein precipitate is collected on a sintered glass filter and thrice extracted by washing with 50 c.c. portions of alcohol-ether. The filtrates are combined and the protein residue is discarded.

The combined alcohol-ether extract is evaporated under reduced pressure at less than 50° C. to a volume of about 30 cubic centimeters. To the substantially aqueous residue, 95

per cent alcohol is added to make a solution containing about 70 per cent alcohol. This solution is extracted three times with equal volumes of petroleum ether. The petroleum ether washings are then discarded.

The clear, aqueous-alcohol extract is evaporated in vacuo to a volume of about 5 cubic centimeters. Fifty cubic centimeters of acetone are added to the residue and 5 drops of saturated magnesium chloride reagent are stirred in. The mixture is allowed to stand in the cold for at least one hour. The clear, supernatant acetone extract is decanted through a sintered glass funnel. The gelatinous precipitate of phosphatide remaining is dissolved in portions of water and 95 per cent alcohol of 3 and 15 c.c. volume respectively. The solution of phosphatide is then evaporated in vacuo to a volume of about 1 c.c., when the precipitation with acetone and magnesium chloride is repeated as described. The acetone extracts are combined and brought to dryness in vacuo. Should the residue from this evaporation have any considerable bulk it should be dissolved in alcohol and water and the precipitation repeated again.

The all but imperceptible residue from the acetone extract is taken up in 50 c.c. of chloroform and chilled. The cold extract is then washed with cold 0.1N. NaOH in the manner described for urine extract. It is then dried by addition of sodium sulfate, filtered through sintered glass, and divided into two samples of equal volume. These samples, to be oxidized and not oxidized respectively, are brought to dryness in 25 c.c. distillation flasks as described.

Colorimetry and calculation are done in the manner described for urine. The result is expressed as milligrams per 100 c.c. of plasma.

RESULTS

Urinary Corticosteroid.—The formaldehyde-forming content of adrenal cortical extract added to samples of urine and carried through the procedure was recovered in a proportion which averaged 80 to 90 per cent. Values obtained from analyses of twelve-hour night urine in ten normal male and female subjects are shown in Table I. Redeterminations on twenty-four hour urine specimens from eight of these subjects show no difference from the mean value found with night urine nor any consistent variations between day and night corticosteroid output. Values found in various abnormal situations and conditions are listed in Table II. Deviations from the normal in the latter series correspond well with clinical prediction. The values found are comparable to those obtained by Daughaday, Jaffe, and Williams,[6] and the methods, as noted, differ only in certain particulars.

TABLE I. AMOUNT OF CORTICOSTEROID-LIKE SUBSTANCE PRESENT IN URINE OF NORMAL MALE AND FEMALE SUBJECTS

MALE SUBJECT	MG./24 HR.	FEMALE SUBJECT	MG./24 HR.
1	0.77	1	1.43
2	1.41	2	.43
3	1.66	3	1.63
4	1.33	4	.76
5	1.29	5	1.09
6	1.00	6	1.36
7	0.94	7	.28
8	0.72	8	.43
9	1.57	9	.32
10	0.81	10	.65
Mean	1.15		0.84
	±0.32		±0.29
Mean for whole group	0.995 mg./24 hr.		

These values were obtained from analysis of twelve-hour night urine and are expressed as milligrame of desorycorticosterone per 24 hours.

TABLE II. OBSERVATIONS ON URINARY CORTICOSTEROID-LIKE SUBSTANCES IN VARIOUS CLINICAL CONDITIONS

PATIENT	SEX	DIAGNOSIS	TREATMENT	RESULT (MG./24 HR.)
1	M	Malignant hypertension		0.91
			Pyrogen 3 days	2.34
			Pyrogen 6 days	4.4
			Pyrogen 9 days	3.5
2	F	Malignant hypertension		1.54
			Pyrogen 1 day	2.27
3	F	Malignant hypertension		1.74
			Pyrogen 1 day	5.1
4	F	Addison's disease	DCA, cortical extract	0.4
5	F	Addison's disease	DCA, cortical extract	0.4
6	F	Addison's disease	DCA, cortical extract	0.4
7	M	Addison's disease	DCA, cortical extract	0.1
8	F	Cushing's syndrome		2.7
9	F	Cushing's syndrome		3.3
			Exploration adrenal	10.8
10	F	Pseudo-hermaphrodite		1.1
11	F	Arrhenoblastoma (?)		1.4
12	F	Acromegaly		0.91

Plasma Corticosteroids.—Recovery of the formaldehyde-forming substances in adrenal cortical extracts added to plasma ranged from 85 to 100 per cent in six determinations. Values found in the venous blood of normal human beings and in normal dogs are listed in Table III. The means in the two groups were 0.25 mg. per 100 c.c. of plasma.

TABLE III. CORTICOSTEROID CONTENT OF BLOOD PLASMA

MALE SUBJECT	MG. PER 100 C.C.	FEMALE SUBJECT	MG. PER 100 C.C.
	Normal Human Beings		
1, a	0.42	1	0.34
b	0.38		
2, a	0.20	2	0.25
b	0.11		
3	0.23	3	0.13
4	0.16	4	0.42
5	0.14		
	Normal Dogs		
0-74	0.38	4-47	0.25
4-45	0.12	5-12	0.12
4-73	0.27	0-12	0.30
4-84	0.21		
5-03	0.38		

Variations of the level were found in normal male human Subject 1 in a value of 0.98 mg. per 100 c.c. thirty-six hours after a second and third degree burn; this value fell to 0.38 mg. per 100 c.c. on the tenth day. Dog 0-12 yielded levels of 0.30 before and 0.6 mg. at twenty-four hours after administration of a bacterial pyrogen. Concurrent urinary corticosteroid values in this animal were 0.15 mg. per twenty-four hours during the three-day period before pyrogen injection and 0.38 mg. per twenty-four hours in the three days after treatment. Variations in plasma level thus correspond with clinical prediction.

The validity of the method is further confirmed in experiments conducted with the assistance of Dr. John Reinhard. In these, the left adrenal efferent vessels of dogs were ligated and a segment of the lumboadrenal vein was isolated

by ligatures where it passes under the gland. A plastic catheter was inserted into the vein and brought out through the abdominal incision which was closed. The operations were done under pentobarbital anesthesia and after administration of heparin. Adrenal venous blood was then collected over varying intervals. Blood loss was partially compensated for by transfusion of whole dog blood, of gelatin, and of washed red blood cells after separation of plasma.

The courses of three such experiments are shown in Fig. 1, which demonstrates the levels of corticosteroid output in the venous plasma of the left adrenal

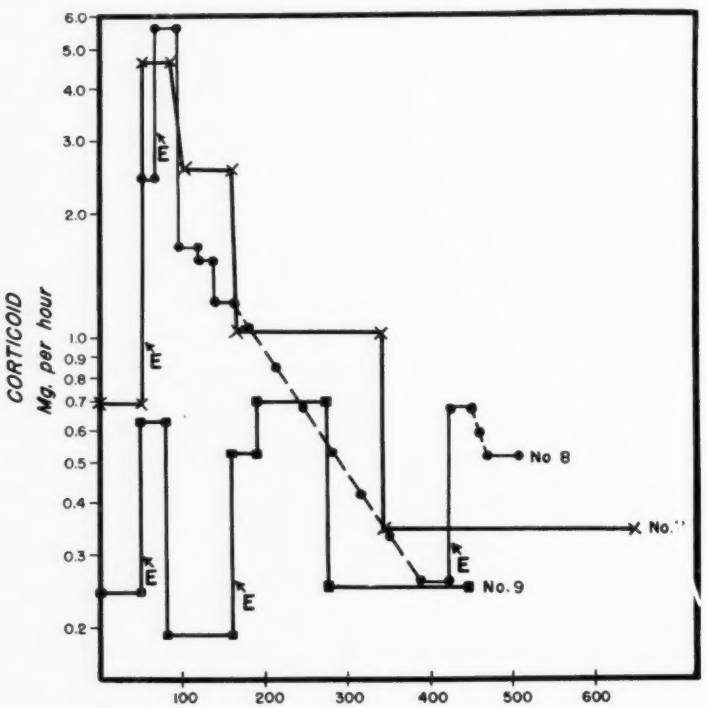

Fig. 1.—Output of corticosteroid (corticoid) into left adrenal venous plasma in three dogs at varying intervals after cannulating the isolated lumbo-adrenal vein. Effect of injection of epinephrine (E); time of injection is indicated by arrows. Abscissa: minutes after starting collection.

TABLE IV. CORTICOSTEROID OUTPUT FROM ADRENAL VEIN IN DOGS; EFFECT OF EPINEPHRINE; PERIPHERAL PLASMA LEVELS

EXPERIMENT	TIME OF COLLECTION (MIN.)		ADRENAL VENOUS PLASMA				PERIPHERAL PLASMA (MG. PER 100 C.C.)		EPINEPHRINE
			CONCENTRATION (MG. PER 100 C.C.)		OUTPUT (MG. PER HR.)				
	(A)	(B)	(A)	(B)	(A)	(B)	(A)	(B)	
3	0-75	76-150	4.8	3.5			0.4	0.9	0.5
3b	0-38	53-70	2.9	3.6	0.64	0.83	0.55	0.76	0.5
7	0-55	60-80	3.3	5.6	0.69	4.7	0.33	0.65	0.5
8	51-61	61-76	2.0	4.45	2.4	5.5	0.93	0.75	0.5
	393-413	414-433	0.24	0.75	0.25	0.69			
9	0-60	61-80	1.0	1.5	0.24	0.63	0.43	0.44	0.1
	160-185	186-216	0.88	1.1	0.19	0.52	0.49	0.7	0.2

Corticosteroid content of adrenal venous plasma at intervals indicated by time of collection in minutes before (A) and after (B) injection of epinephrine into the femoral vein. Output is calculated by multiplying plasma concentration by adrenal venous plasma flow.

gland in milligrams per hour and the effects thereon of administration of epinephrine in doses of 0.2 to 0.5 mg. into the femoral vein. Concentrations of corticosteroids in adrenal and peripheral venous plasma and the steroid outputs as well as simultaneous measurements in peripheral blood are listed in Table IV. The concentrations found in the adrenal venous plasma were higher than those obtaining in peripheral blood. The steroid output in these experiments averaged 0.85 mg. per hour at the outset. In four of six experiments in which measurements were made, the increased corticosteroid output caused by epinephrine was reflected in an increase in the concentration present in peripheral blood. However, we direct attention to these experiments (which will be reported in more detail) to confirm the method proposed rather than to deal with physiologic variations in the adrenal steroids.

DISCUSSION

Urinary Corticosteroid.—Acidification of urine before extraction follows the recommendation of Heard, Sobel, and Venning.[7] Extraction is carried on four times to yield satisfactory recoveries of added adrenal cortical extract. Thus, recoveries with two, three, and four extractions are 31, 65, and 94 per cent respectively.

The crude chloroform extract is washed with sodium hydroxide in the manner of Talbot, Salzman, Wixom, and Wolfe.[8] The washing reduces the bulk of the residue and thus reduces the probability of nonspecific contamination of the material which is to be oxidized. Other data[5] suggest that this step is not essential. However, on several occasions the residue from washing with water and alkali has been shown to contain small but significant amounts of chromogenic material.

The residue from the washed chloroform extract is dissolved in glacial acetic acid because of the good wetting quality of this reagent. The residue is not partitioned with benzene and water as was proposed[1] or as recently described[5] because of the incompleteness of water extraction of certain steroids from benzene (Heard and Sobel[9]) and because the benzene-water partition of adrenal cortical extract yields a ratio of color density in benzene as compared with water of about 0.65.

Distillation of formaldehyde is done because of interfering colors which form during the heating of urinary or adrenal cortical extracts in the strong acid of the chromotropic acid reagent. The distillate is not received in sulfite as in the method of Daughaday, Jaffe, and Williams[5] because formaldehyde can be quantitatively recovered in water. It is important to distill a sufficient volume. With distillate volumes of 4.5, 5.0, 5.5, 8.0, and 8.5 c.c., the respective recoveries of formaldehyde are 63, 75, 78, 98, and 101 per cent.

Mason* developed an almost identical procedure. In his method, as in ours, fractionation of the steroid extract with Girard T reagent[8] is omitted because results on unfractionated extracts parallel those obtained with the more tedious fractionation. It may be of interest to note that, in our hands, the ketonic

*We acknowledge the cooperation and advice of Dr. H. L. Mason, Mayo Clinic, Rochester, Minn.

fraction in urinary extracts accounted for about one-third of the total oxidizable steroid.*

Plasma Corticosteroid.—The desirability of determinations of plasma corticosteroid is suggested by the fact that the amount measured in human urine averages about 0.05 per cent of the amount circulating through the kidneys. Rephrased, the urinary plasma clearance of corticosteroid is only 0.11 c.c. per minute. The apparent threshold of excretion is therefore very high, as indeed it should be for a material so important in bodily function. However, the inference is that under certain conditions measurements of plasma corticosteroid may have greater diagnostic and physiologic value than measurements in urine. This is especially true in experiments of brief duration, such as after injection of epinephrine.

The feasibility of such a determination was demonstrated by Hemphill and Reiss.[10] Their procedure measured corticosteroid by applying to plasma extracts the method of Talbot, Salzman, Wixom and Wolfe,[8] while the method here presented has the advantages of the procedure described for urine extracts. As compared with the method of Hemphill and Reiss[10] the extraction process has been elaborated. Thus, we have found it essential to precipitate out phosphatide and, apparently because of entrainment of corticosteroid onto the phosphatide precipitate, the precipitation has to be repeated more than once. Further, an interfering chromogen is separated by washing plasma extract in 70 per cent alcohol with petroleum ether. Preliminary experiments indicate that it may be more convenient to make the first extraction by adding plasma to acetone containing 2 per cent chloroform. In a personal communication, Dr. O. M. Hechter† suggests that direct extraction of whole blood or plasma with chloroform is also satisfactory. Thus, while it is apparent that modifications can be made in the procedure, it is presented here in the form in which it has been most extensively tested.

As noted previously, observations on the corticosteroid output of the adrenal in dogs and the effects thereon of adrenalin are not the focus of this communication. Still it is of interest to note that the experiments listed here confirm the observations Vogt has made by bio-assay.[11] Thus, she estimated output of active steroid at about 230 c.c. Eucortone per twenty-four hours in a dog weighing 10 kilograms. Our estimate in terms of Upjohn adrenal cortical extract analyzed by oxidation and colorimetry would be about 88 cubic centimeters. Again, Vogt showed an increase of hormone output after injection of epinephrine. This estimate is confirmed in Fig. 1. In Table IV we also show that increased output due to epinephrine is often reflected in an increase in the corticosteroid content of peripheral as well as adrenal plasma.

As might be surmised, simple modifications enable the application of the plasma procedure to samples of tissue.

SUMMARY

Methods are presented for the estimation of corticosteroids in urine and plasma. The underlying principles are (1) extraction and partial separation

*We would also note the helpful interest of Dr. Eleanor Venning of the Royal Victoria Hospital, Montreal, Canada.

†Worcester Foundation for Experimental Biology, Shrewsbury, Mass.

of neutral steroids from urine or plasma, (2) oxidation of the side chains of the C_{21} steroids of adrenal cortical origin by means of periodic acid, and (3) colorimetric measurement of formaldehyde formed in the oxidation. Values found in normal subjects are listed, together with illustrative clinical and experimental observations which indicate the application of these procedures.

The authors take pleasure in noting the skillful assistance of Mr. Frank Ungar, B.A., M.S., in the development of the plasma procedure, and of Mrs. Lorraine Friedman, B.A., in the urine method.

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QUICK MICROTECHNIQUES FOR THE IDENTIFICATION OF CULTURES

I. Indole Production

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THE bacteriologist has always been handicapped in identification of organisms, as compared with other biologists, by the fact that the organisms with which he deals cannot be identified by their morphologic characteristics alone. The necessity of including cultural and biochemical characteristics has made identification a time-requiring procedure. Cultures need time to grow and to produce biochemical reactions.

In the diagnostic laboratory especially, the value of bacteriologic work definitely has been limited by the time required to isolate and identify the organisms from the patient. In addition, bacteriologic work often becomes expensive because of the time required and the materials and equipment needed. The chemist has partially solved the problem of expense by the development of semimicro- and microtechniques.

This study of indole production was undertaken to see if microtechniques could be applied to the study of biochemical reactions of microorganisms and if, at the same time, results could be obtained in a shorter period of time.

The principle on which the test is based is the use of small amounts of media and large inoculums to allow the organisms to start growing without appreciable lag phases and to produce, in a short period of time, sufficient concentrations of biochemical products to be detectable if sensitive reagents are used. Studies have been made to determine the optimum conditions for indole production and detection by the microtechnique.

DEVELOPMENT OF THE MICROTECHNIQUE

Tests proved to be most satisfactory with 1 ml. quantities of medium in 10 by 75 mm. tubes. Smaller tubes were not practicable because of difficulty in mixing the test solution with the medium.

For the detection of the indole, the Ehrlich, Kovács,[1] and Goré[2] methods were tried. For the preliminary testing of these methods a medium containing 1.0 per cent tryptone and 0.3 per cent beef extract was used. Tubes of this medium were heavily inoculated with a stock strain of *Escherichia coli*. Tests for indole were performed on these tubes after periods of incubation at 37° C. for from two and one-half to six hours. With the Kovács method a doubtful reaction was obtained in one of two tubes after three hours of incubation, and a strongly positive reaction was obtained in all the tubes that were incubated for four hours or longer. The other two methods were less sensitive, requiring

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at least one hour more of incubation to obtain comparable results. Because of these results and because its application is simplest, Kovács' method was selected for all subsequent work.

In the test, four drops of fresh Kovács reagent are added to the 1 ml. quantity of medium in the tube. The tube is shaken and the results are read after a few minutes. In the early part of the work the chloroform solubility test of Fellers and Clough[3] was used to check the specificity of the positive reactions. Since these reactions always proved to be specific, this test was discontinued later. The Kovács reagent must be fresh. Upon standing at room temperature its color changes from yellow to brown and it becomes less sensitive. It may be preserved for several days in the refrigerator. For the preparation of the reagent, various brands of amyl alcohol, isoamyl alcohol, and isobutyl alcohol were tried. Of these, the isoamyl alcohol proved to be superior, some of the amyl alcohols giving false colorations and the isobutyl alcohol failing to separate sharply from the medium.

After a study of a number of media, two were selected for use in the microtechnique. Medium 1 consisted of 1.0 per cent tryptone and 0.3 per cent beef extract, in distilled water. Medium 2 consisted of 0.03 per cent tryptophane, 0.1 per cent peptone, and 0.5 per cent K_2HPO_4, in distilled water. It was found that in Medium 1 a salt mixture consisting of 0.5 per cent NaCl, 0.02 per cent $MgSO_4$, 0.01 per cent $CaCl_2$, and 0.1 per cent K_2HPO_4 could be substituted for the beef extract without affecting the results. Since the salt mixture added to the complexity of the medium without adding to its productiveness, it was not used in later tests of the technique. The addition of either beef extract or the salt mixture to Medium 2 did not affect the results obtained. For the peptone in Medium 2, Coleman and Bell Bacto-peptone, Bacto-proteose peptone 3, Bacto-tryptone, and Bacto-tryptose were tried. All gave equally good results.

The addition of 0.6 per cent agar to Medium 1 caused false colorations to be produced with the test reagent. A medium consisting of 1.0 per cent Bacto-protone and 0.3 per cent beef extract was found to be unsuitable for use in the microtechnique.

Batches of Medium 1 and of Medium 2 with pH values varying from 7.0 to 8.0 were tried. Equally good results were obtained with pH values between 7.4 and 7.8. At pH 7.0 and pH 7.2 indole was formed as quickly as at the higher pH values, but the amount formed was less. For example, in one experiment indole was formed at all pH values between 7.0 and 7.8 in six minutes, either doubtful or weak reactions being obtained. After fifteen minutes of incubation, all tubes with pH values between 7.4 and 7.8 yielded strongly positive reactions whereas those with pH values of 7.0 and 7.2 yielded doubtful or weak reactions. In the later use of the microtechnique all media were adjusted to pH 7.4.

The testing of a large number of cultures with Medium 1 and Medium 2 indicated little difference in value for the two media. No discrepancies in results have been noted. With many cultures the time required for indole production in Medium 2, from tryptophane, is about one third less than the time required

for production in Medium 1, from tryptone. This saving in time may not be considered sufficient to warrant the use of the more expensive Medium 2.

Quick production of indole depends upon rapidly obtaining the incubation temperature of 37° C. Preheating of the tubes of medium before inoculation shortens materially the time required for indole production. Water bath incubation instead of hot air incubation also helps. Usually if a culture will produce indole in two hours without preheating of the medium it will do so in from thirty minutes to one hour with preheating and in from eighteen to thirty minutes with water bath incubation. We have obtained indole production in six minutes with water bath incubation.

Absolute sterility has been found to be unessential with the microtechnique. In this study the tubes and the medium were always sterile but a relative absence of contaminants is probably all that is necessary. Cotton plugs do not need to be used. A small number of contaminants cannot multiply rapidly enough to affect the results of the test.

The size of inoculum is important. Inoculation of a tube with all the growth obtainable from a colony with a diameter of 2 mm. will give good results. Inoculation with the larger amount of growth obtainable from an agar slant culture will yield somewhat quicker indole production. Inoculation with growth from a culture which is in the logarithmic period of development will give quicker results than inoculation from an older culture.

COMPARISON OF MICROTECHNIQUE WITH GNEZDA TECHNIQUE

For testing the accuracy of results obtainable with the microtechnique, comparative tests were made with the Gnezda[4] technique. For the Gnezda technique tryptone broth was used and incubation was at 37° C. for four days.

Results with the two techniques were identical. Cultures which gave positive results were fifty-seven strains of coliforms, three strains of *Pseudomonas caviae,* two strains of *Proteus mirabilis,* and one strain each of *Proteus vulgaris* and *Shigella paradysenteriae.* Of these, two of the strains of coliforms yielded weakly positive results with the Gnezda technique after four days. All the other strains gave strongly positive results with both methods. Cultures which gave negative results were three strains of coliforms, nine strains of unidentified soil organisms, and one strain each of *Salmonella schottmülleri, Salmonella paratyphi, Salmonella enteritidis, Vibrio comma, Pseudomonas aeruginosa,* and *Pseudomonas fluorescens.* The majority of the strains of coliforms were fresh isolates and the tests were made with inoculums from the primary colonies on the selective media used for isolation. All other cultures used were stock strains.

The rapidity with which indole was formed in the microtechnique raised the question as to whether the indole was being formed because of growth of the organisms and enzyme production during incubation or because of preformed enzymes that were present in the large inoculum. In this connection we were interested in determining the effect of the medium from which inoculum was taken on the rate of indole formation.

From thirteen strains of coliform organisms, streaks were made on nutrient agar plates and on tryptone agar plates. After a twenty-four hour period of

incubation, tests for indole-forming ability were made by the microtechnique with inoculums from both types of plates. Of the thirteen strains, ten gave positive indole tests after one and one-half hours when the inoculums were taken from the nutrient agar plates. Of these, one gave a positive test after eighteen minutes, five after thirty-six minutes, two after one hour, and two after one and one-half hours when the inoculums were taken from the tryptone agar plates. Thus, growth of the inoculum culture on tryptone agar appeared, in the majority of cases, to increase the speed with which indole was produced.

Using the two strains of coliforms that had produced only traces of indole after two hours, an attempt was made to increase the rate or amount of indole formation by transferring for two generations on tryptone agar plates. No increase in rate or amount of indole formation occurred.

The laboratory strain of *Escherichia coli* was grown for four generations on a synthetic tryptophane-free medium. At the end of this time indole determinations were made using colonies from the synthetic medium, from nutrient agar plates, and from tryptone agar plates as sources of inoculum. Again indole was formed more rapidly in the tubes that had been inoculated from colonies on the tryptone agar plates. Surprisingly, however, indole was formed slightly more rapidly in the tubes that had been inoculated from the synthetic medium than in those that had been inoculated from the nutrient agar plates.

From this series of experiments it may be concluded that the medium from which the inoculum is taken has only a minor effect on the results of the microtechnique for the determination of indole-producing abilities of bacterial cultures.

SUMMARY

A quick microtechnique for the determination of the abilities of microorganisms to produce indole has been described. Heavy inoculations are made into 1 ml. quantities of medium in 10 by 75 mm. tubes that have been preheated to 37° C. Either tryptone broth or a synthetic medium containing tryptophane has been found to be satisfactory. The tubes are then incubated at 37° C., preferably in a water bath. Indole may be detected in the tubes upon the addition of 4-drop quantities of a fresh Kovács solution which has been made with isoamyl alcohol as the solvent. The period of incubation that is necessary has been found to vary from six minutes to two hours, depending upon the strain of organism, the age and size of inoculum, and the method of incubation.

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QUICK MICROTECHNIQUES FOR THE IDENTIFICATION OF CULTURES

II. Fermentations

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SUCCESS in developing a quick microtechnique for the detection of indole-producing abilities of microorganisms (Arnold and Weaver[1]) led to an attempt to develop a similar technique for the demonstration of fermenting abilities of microorganisms. The principle on which the test to be reported is based is the same as that used in the test for indole production, namely the use of small amounts of media and large inoculums to allow the organisms to start growing without appreciable lag phases and to produce, in a short period of time, sufficient concentrations of biochemical products to be detectable if sensitive reagents are used.

DEVELOPMENT OF THE MICROTECHNIQUE

The microtechnique was developed using cultures of the Enterobacteriaceae as test organisms. Thus far its use has been limited to this group of organisms.

Tests proved to be most satisfactory with 0.15 ml. quantities of medium in 5 by 50 mm. tubes. Larger tubes, 10 by 75 mm., containing 0.5 to 1.0 ml. quantities of medium could be more easily handled, but their use in place of the smaller tubes approximately doubled the time required for the demonstration of fermentation.

Of a number of basal media that were investigated, all but one was discarded as unsatisfactory. Thioglycollate broth, nutrose solution, neopeptone solution, and neopeptone and tryptone solution were discarded because the rate of fermentation in them was too slow. A yeast extract medium was discarded because the yeast extract contained some substance or substances that would allow the production of both acid and visible gas by *Escherichia coli* cultures under the conditions of the microtechnique.

The following basal medium was selected for use. Difco beef heart infusion, 7.5 per cent; proteose peptone 3, 1.0 per cent; KH_2PO_4, 0.1 per cent; NaCl, 0.5 per cent; distilled water; pH 7.0. Indicator: 5 ml. of a 1.6 per cent alcoholic solution of bromcresol purple and 5 ml. of a 1.6 per cent alcoholic solution of cresol red per liter of medium.

This medium proved to be free of fermentable substances when it was tested with strains of *E. coli*. Later it was found that controls of this basal medium that had been inoculated with *Aerobacter aerogenes* showed slight visible gas formation, although by the usual macrotechniques *A. aerogenes* could not be shown to produce any fermentation in the medium. It was therefore necessary

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to remove the fermentable substances from the beef heart infusion by fermentation with *A. aerogenes*. The beef heart infusion was dissolved in the distilled water. This solution was inoculated with 5 ml. of a twenty-four hour nutrient broth culture of *A. aerogenes*. It was then incubated at 37° C. until bubbles ceased to appear, approximately forty hours. The organisms were removed by passage through a filter pad.* The remaining ingredients were then added to this infusion, and the resultant medium was adjusted to pH 7.0. This basal medium was then sterilized by autoclaving.

When the tests were to be run, a sufficient quantity of a 20 per cent solution of the carbohydrate to be tested to produce a final concentration of 5 per cent was added to the basal medium. It was found that the carbohydrate solution had to be sterilized by filtration. The use of autoclaved solutions resulted in false reactions by the microtechnique even when the use of the same solutions in medium for tests by the macrotechnique did not. The final medium was then dispensed in the small tubes with capillary pipettes. In all the work reported upon in this study sterile pipettes and sterile tubes were used, but it has been found that only relative sterility is necessary. Cotton plugs may be omitted. It was found that the time required for the test could be reduced by preheating the tubes of medium in the water bath previous to inoculation.

If inoculation is to be with liquid-medium cultures or with suspensions of the organisms in saline, the medium should be prepared in double strength to allow for dilution of the medium with the liquid of the inoculum.

The inoculum should be large. The time required for the test will be shortened if the culture from which the inoculum is taken is in the logarithmic growth period. Inoculation from growth on a solid medium with a platinum needle is satisfactory. In this study most of the inoculations were made with suspensions that had been prepared by emulsifying growth from an agar slant culture or from a colony in a small amount of sterile saline. Inoculations were made in this case by adding 0.15 ml. quantities of the suspensions to the medium in the tubes by means of capillary pipettes.

The inoculated medium was then capped with a 3 mm. layer of 1 per cent agar in distilled water, to which indicator had been added in the proportion of 1 ml. each of a 1.6 per cent alcoholic solution of bromeresol purple and a 1.6 per cent alcoholic solution of cresol red per liter. The indicator was added to the agar solution to detect any change in pH due to absorption of acid substances from the air during storage. When this precaution was not taken, the reading of the results was occasionally confused by a small area of acid reaction at the junction of the agar solution and the culture caused by an agar solution that had become acid. Less indicator was added to the agar than to the medium to make the junction of these solutions more discernible.

The capping is done with a capillary pipette. The melted agar is permitted to flow down the inside wall of the tube. Care must be taken to avoid bubbles. If bubbles form they can usually be eliminated from the agar-medium interface by a gentle tapping of the tube.

*Seitz Serum No. 1.

The quickest results are obtained by incubation in a 37° C. water bath. This is due to the fact that the medium reaches the incubation temperature much more quickly in the water bath than it does in a hot-air incubator.

Acid production may first be noted by the production of a yellow color just beneath the agar cap. Readings must always be made by comparison with inoculated controls of the basal medium that do not contain the test carbohydrate. If this is not done, false results may be obtained because of transference of acid to the medium with the large inoculum. Gas production is evinced by the collection of bubbles at the agar-medium interface. Acid production usually may be detected before gas production, but the opposite is occasionally the case.

RESULTS WITH MICROTECHNIQUE

Results with the microtechnique were compared with those with the macrotechnique on five strains of *A. aerogenes*, five strains of *E. coli*, ten strains of paracoli, ten strains of Salmonella, three strains of *Eberthella typhosa*, five strains of Shigella, and seven strains of Proteus using glucose, mannitol, sucrose, lactose, and maltose. The macrotechnique was run using Durham fermentation tubes and nutrient broth base plus filtered sugar solutions. Final results were read after seventy-two hours. Two determinations were made with the microtechnique. In the first, the tubes were inoculated with suspensions obtained by emulsifying single colonies from nutrient agar plates in 1 ml. quantities of sterile distilled water. In the second, the tubes were inoculated with heavy suspensions obtained by emulsifying growth from twenty-four hour cultures on brain-veal agar slants.

The essential features of the results are summarized in the following statements.

Identical results were obtained with the macrotechnique and with both sets of microtechnique determinations.

The use of a heavy inoculum materially shortens the time required to obtain results with the microtechnique. With the light inoculum, acid production was obtained in from 15 to 725 minutes and gas production in from 75 to 660 minutes. With the heavy inoculum, acid production was obtained in from 10 to 230 minutes and gas production in from 35 to 240 minutes.

The simpler fermentable substances were fermented more rapidly than were the disaccharides. For example, with the six strains of organisms that fermented all the substances that were tested and with the light inoculum, an average of 95 minutes was required for production of acid from glucose, 85 minutes from mannitol, 130 minutes from sucrose, 140 minutes from lactose, and 145 minutes from maltose.

COMMENT

The microtechnique yields results that are as reliable as those obtained with the usual macrotechniques. It not only yields quicker results but its use also results in a considerable saving in time and materials. The stock basal medium may be sterilized and stored in the refrigerator. Likewise, 20 per cent solutions

of the various fermentable substances may be sterilized by filtration and stored in the refrigerator, with proper precautions being taken against evaporation. When it is desired to test cultures, the fermentable substances and basal medium may be mixed in the proper proportions and the tests may be performed immediately. Only precautions to prevent excessive contamination in the process are necessary.

The microtechnique is usable under a variety of conditions. The length of the incubation period depends upon these conditions. The most important condition is the type and size of inoculum. The quickest results are obtained with heavy inoculums with logarithmic growth phase cultures that have been grown on a rich medium. On the other hand, reliable results can be obtained with single colonies from primary isolation plates. While the incubation period required in the microtechnique in the latter case is longer, the results are obtained much more quickly than if it were necessary to grow secondary cultures before the microtechnique tests were performed.

SUMMARY

A quick microtechnique for the demonstration of fermenting abilities of microorganisms has been described. It has proved to give reliable results with members of the Enterobacteriaceae. No tests have been run on other cultures.

Heavy inoculations are made into 0.15 ml. quantities of medium in 5 by 50 mm. tubes which have been preheated to 37° C. A beef heart infusion medium containing indicator is used as the basal medium. To it is added a sufficient quantity of sterile (filtered) 20 per cent solution of the fermentable substance to be tested to produce a 5 per cent solution in the final medium. The inoculated medium is capped with a 3 mm. layer of a melted 1 per cent agar solution. The tubes are incubated at 37° C. Gas production is evinced by the collection of bubbles below the agar cap. Incubation periods of from 10 minutes to 12 hours, depending upon the size of inoculum and other factors, have been found to be necessary.

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A TEST OF THE COAGULATION TIME OF BLOOD HEPARINIZED IN VITRO; STUDIES OF NORMAL SUBJECTS AND OF PATIENTS WITH INTRAVASCULAR THROMBOSIS

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IT IS known that the addition of a specific amount of heparin to a specific amount of human blood may produce a variable increase of the coagulation time among different individuals. This principle has been applied by others in an attempt to develop a test which might be of value as an indication of increased coagulability of the blood in patients who have or may have thrombosis.

In 1943 de Takats and Gilbert[1] described a heparin tolerance test. They injected 10 mg. of heparin intravenously and determined the coagulation time of capillary blood drawn into capillary tubes before and at ten-minute intervals after injection until the coagulation time returned to preinjection levels. With this test de Takats[2] noted that little or no increase of coagulation time occurred in patients who had undergone major surgical procedures or who had had coronary thrombosis, venous thrombosis, arterial embolism, or thromboangiitis obliterans, while a definite increase occurred in normal persons. Although they do not detract from the importance of the principle involved, there are certain theoretic objections to the technique which was used; namely, the method of testing the coagulation time that de Takats and Gilbert used is not generally considered to be as accurate and reproducible as other methods, the differences between the maximal coagulation times in normal persons and those with thrombosis were of the order of only a few minutes and possibly within the range of error of the method (Meyer),[3] and tests were done at room temperature.

Waugh and Ruddick[4, 5] in 1944 described a test of coagulation time of blood heparinized in vitro. Their method consisted of adding 1 c.c. of venous blood to each of a series of test tubes in which there were increasing amounts of heparin from 1 unit (0.009 mg.) to 7 units (0.063 mg.). The chief practical objections to this method are that it is time consuming and that too many tubes requiring accurate mixtures of blood and heparin are necessary for each test. Waugh and Ruddick did their tests at room temperature.

Hagedorn[6] repeated de Takats' heparin tolerance test but administered a larger amount of heparin, 25 mg., and determined coagulation times on venous blood (Lee-White method). He found that only two coagulation times were important, namely the one before injection and the one ten minutes after injection. Significant differences were found between the responses of normal

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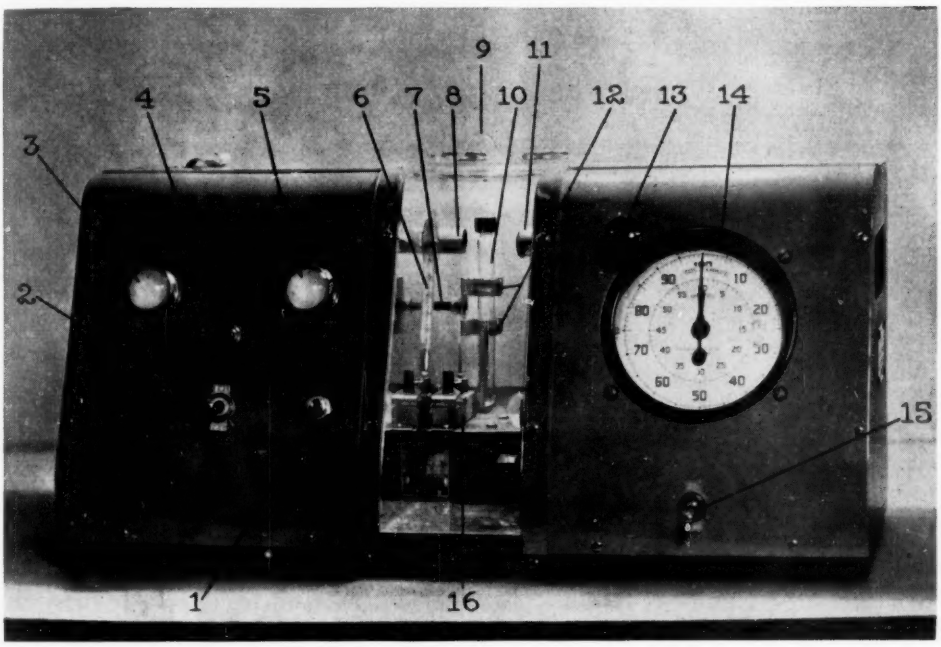

Fig. 1.—Front view of coagulochronometer: *1*, access to adjusting screw for varying sensitivity of photoelectric cell; *2*, motor switch; *3*, green light; *4*, push button to permit continuous rotation of test tube; *5*, red light; *6*, thermometer; *7*, motor shaft; *8*, tube transmitting beam of light to photoelectric cell; *9*, handle of plastic door permitting access to chamber for insertion and removal of test tube; *10*, test tube to hold blood being tested; *11*, tube to transmit beam of light from source of light; *12*, clamp for test tube; *13*, lever to reset stop clock; *14*, electric stop clock; *15*, switch for stop clock; *16*, heating unit and thermostat.

persons and the responses of a series of patients with intravascular thrombosis of various types. The differences seemed much greater than the possible error of the method of determining the coagulation time. Hagedorn also found a close correlation between the coagulation time of venous blood ten minutes after the intravenous injection of 25 mg. of heparin and the coagulation time of 1 c.c. of venous blood added to a solution of 0.005 mg. of heparin in vitro in each of a large series of both normal persons and patients with thrombosis. The chief objections to the Hagedorn method were that each test was time consuming and that tests were done at room temperature.

We wish to report the results of a coagulation test of venous blood heparinized in vitro in which we have attempted to increase the accuracy of the method and at the same time to simplify the technique so that only a minimal amount of time is expended in the performance of each test. In this test we have utilized the following equipment: a Barker coagulochronometer, a syringe adapted for accurately measuring and immediately mixing blood with a heparin-saline solution, a box to maintain a constant air temperature of 37° C., Pyrex test tubes, venipuncture needles, and a solution of heparin.

The Barker coagulochronometer[7] (Fig. 1) is a machine that automatically records the coagulation time of whole blood or heparinized whole blood. The machine consists essentially of a photoelectric cell which operates a sensitive

relay, a light source for the cell, an electric motor which rotates at the rate of 1 revolution per minute, a horizontal shaft fitted with a test tube clamp, and an electric stop clock. Blood is placed in a test tube which is closed with a rubber finger cot or cork and the test tube is placed in the clamp where it is grasped by its midportion. As the test tube rotates, its top passes through the beam from the light source. As long as the blood is fluid, it runs to the lower end of the tube with each half-revolution. When the blood coagulates it adheres to the lower end of the tube, and when that end of the tube rotates upward to the vertical position, the blood clot interrupts the light beam and breaks the electrical circuit operating the motor and stop clock. Thus the coagulation time is automatically recorded. This machine is primarily a laborsaving device, since it eliminates frequent tipping of the tube by hand to determine the end point. Also it tips the tube at a constant rate and to a constant degree.

To insure direct and accurate mixing of the heparin and blood, a syringe was devised by one of us.[8] This syringe, when fitted with a 21 gauge, 1½ inch (3.8 cm.) venipuncture needle, holds exactly 1 c.c. of heparin in a saline solution. Venipuncture was then performed with this syringe. By means of a spring release mechanism, exactly 1 c.c. of blood could be withdrawn into the heparin-saline solution. The blood and the heparin solution were mixed immediately in the syringe.

Whittaker[9] demonstrated that heparinized blood must be kept at a constant temperature to obtain accurate coagulation times. In our early studies the coagulochronometer was kept in a cabinet in which the air temperature was maintained at 38° C. Later the midportion of each coagulochronometer was enclosed in transparent plastic and the air surrounding the test tube containing the blood was kept at 37° C. by means of a heating element, thermostat, and fan inside the closed space.

The solution of heparin was prepared by diluting Abbott's solution of heparin (10 mg. per cubic centimeter) with 0.9 per cent solution of sodium chloride so that 1 c.c. contained 0.006 mg. of heparin. This solution was prepared under sterile conditions and stored in vaccine bottles sealed with rubber diaphragms. All glassware was thoroughly cleaned and dried in drying ovens. When not in use, equipment was covered to avoid contamination with dust particles.

In performing a coagulation test with heparinized blood, a 21 gauge, 1½ inch (3.8 cm.), steel venipuncture needle was attached to the syringe and 1 c.c. of heparin-saline solution was drawn into the barrel of the syringe. A tourniquet was applied to the subject's arm and as soon as one of the cubital veins became distended it was punctured. A finger release spring on the syringe was then depressed and the plunger was pulled back until it stopped. In this way exactly 1 c.c. of whole blood was added directly to the 1 c.c. (0.006 mg. of heparin) of heparin-saline solution. Care was taken that the venipuncture was neat and that no air entered the heparin-saline-blood mixture. The tourniquet was released and the needle was withdrawn from the vein. The needle was removed from the syringe and the contents of the syringe were gently poured

down the side of a Pyrex glass tube 8 mm. in diameter and 100 mm. long. The test tube was sealed with a rubber finger cot and gently inverted to mix its contents. The test tube was then placed in the test tube holder of the coagulochronometer and the time clock and the motor were started. When the blood coagulated, it broke the light beam, thus stopping the motor and the clock. The coagulation time was read from the clock dial. Hereafter in this paper the time is referred to as the heparin coagulation time.

After the blood had been withdrawn from the vein, there was a delay of about one minute before it could be placed in the machine and the time clock started. Since this delay was short and a relatively constant factor in all of our tests, we do not feel that it constitutes a significant source of error in interpretation of results.

It was our experience in determining coagulation times with this technique that if coagulation did not occur within thirty minutes, a satisfactory end point rarely occurred. It appeared that the constant rotation of the tube ultimately produced defibrination of the blood. Thus the results of all the tests could be roughly divided into two groups: those in which coagulation occurred in less than thirty minutes and those in which coagulation did not occur in thirty minutes.

To determine the variability of the heparin coagulation test, repeated tests were done on each of sixteen subjects in whom the first heparin coagulation time was less than thirty minutes. In each subject three successive separate blood samples were drawn according to the described technique and each sample was placed in a different coagulochronometer. The results are shown in Table I. The variability measured as the standard deviation is shown

TABLE I. RESULTS OF THREE SUCCESSIVE HEPARIN COAGULATION TESTS DONE ON THE SAME INDIVIDUAL WITH THREE SUCCESSIVE VENIPUNCTURES*

SUBJECT	HEPARIN COAGULATION TIME (MIN.)				
	TEST			MEAN	STANDARD DEVIATION
	1	2	3		
A	13	14	18	15.0	2.6
B	14	11	17	14.0	3.0
C	14	12	17	14.3	2.5
D	20	24	18	20.7	3.1
E	25	28	22	25.0	3.0
F	18	12	19	16.3	3.8
G	15	16	15	15.3	0.6
H	10	14	15	13.0	2.6
I	18	24	20	20.7	3.1
J	17	20	25	20.7	4.0
K	25	17	26	22.7	4.9
L	20	22	19	20.3	1.5
M	16	18	15	16.3	1.5
N	12	11	20	14.3	4.9
O	25	27	NC†	26.0	1.4
P	17	15	18	16.7	1.5
Total series				18.0	2.7

*Subjects in whom the first determination of coagulation time was less than thirty minutes.

†No coagulation.

for each case. For the sixteen subjects the average standard deviation was 2.7 minutes, giving a significant variability of plus-minus 5.4 minutes.*

Five successive blood samples were drawn from five individuals in whom the first heparin coagulation time was more than thirty minutes. In none of the subsequent tests on these subjects was the heparin coagulation time less than thirty minutes.

The results of our studies of heparin coagulation time in control groups and in diseased states are shown in Table II. The second group in Table II consisted of patients who had been in bed from six to seventy days because of various diseases, but who did not have clinical evidence of intravascular thrombosis. In the cases of thromboangiitis obliterans the disease was severe enough to require hospitalization. In each of the cases of arteriosclerosis obliterans the patient had been hospitalized because of ischemic ulcers or gangrene, but in no case had there been a recent arterial occlusion.

TABLE II. DISTRIBUTION OF HEPARIN COAGULATION TIME IN VARIOUS CONDITIONS

DIAGNOSIS	TOTAL INDIVIDUALS TESTED	HEPARIN COAGULATION TIME (MIN.)							
		30 or longer		20-29		10-19		LESS THAN 10	
		NUMBER	PER CENT	NUMBER	PER CENT	NUMBER	PER CENT	NUMBER	PER CENT
Normal ambulatory subjects	50	41	82	2	4	7	14	0	
Bed patients without thrombosis	50	39	78	4	8	7	14	0	
Thromboangiitis obliterans	14	8	57	2	14	4	29	0	
Arteriosclerosis obliterans	20	8	40	3	15	9	45	0	
Recent venous thrombosis or pulmonary embolism	19	2	11	5	26	2	11	10	52
Acute peripheral arterial occlusion	3	1	33	0	0	0	0	2	62

In each case of thrombophlebitis or pulmonary embolism in Table II, the diagnosis was definitely established clinically and blood samples were drawn within one to seven days after the clinical onset of thrombosis. Fifty-two per cent of these patients had coagulation times less than ten minutes; this rapid coagulation time was not observed in any of the control subjects whom we studied.

We also had an opportunity to study three patients with acute arterial occlusion in the extremities shortly after the occlusion occurred. In one patient the heparin coagulation time was thirty minutes or more, but in two the time was less than ten minutes.

SUMMARY

We have described a technique for the determination of the coagulation time of 1 c.c. of venous blood mixed with 1 c.c. of 0.9 per cent solution of sodium chloride containing 0.006 mg. of heparin. The technique utilized a

*Plus-minus 2 times the standard deviation is a generally accepted range for statistically significant variation.

coagulochronometer (a machine for automatically recording the end point on a stop clock) and a syringe which was specially devised to permit first the withdrawal of exactly 1 c.c. of the heparin-saline solution from a bottle and then of exactly 1 c.c. of blood from a vein into the heparin-saline solution. All tests were done in an environmental temperature of 37° C.

Coagulation times by this method were less than thirty minutes in only 18 per cent of fifty normal ambulatory subjects and in 22 per cent of fifty bed patients without evidence of thrombosis. Coagulation times were less than thirty minutes in 53 per cent of thirty-four patients with chronic occlusive arterial disease of the extremities (thromboangiitis obliterans or arteriosclerosis obliterans). Coagulation times were less than thirty minutes in 89 per cent and less than ten minutes in 52 per cent of nineteen patients with recent clinical venous thrombosis or pulmonary embolism. No coagulation times were less than ten minutes in any of the control subjects.

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A CAGE WHICH LIMITS THE ACTIVITY OF RATS

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THE cage shown in Fig. 1 was developed to restrain a rat during periods of days while an indwelling tube was in place. It has proved very useful in collecting urine through an indwelling cystostomy tube, for continuous or intermittent injection through a small plastic tube inserted into a vein, for collection of lymph from the thoracic duct, intestine, or liver over periods of days through a small indwelling plastic tube in a lymphatic vessel, and for other procedures.

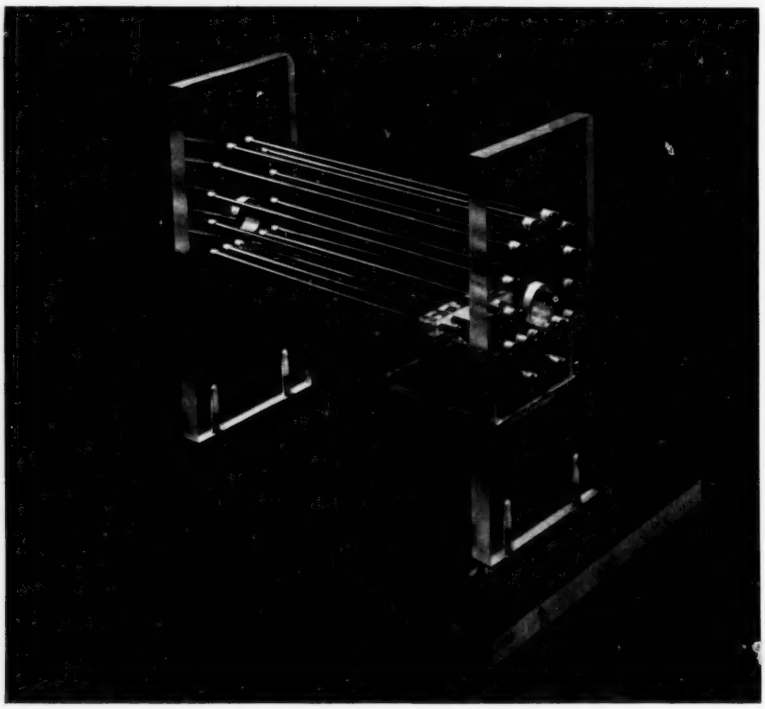

Fig. 1.—The assembled cage.

The cage is made of Lucite $\frac{3}{8}$ inch thick, which may be worked with ordinary tools. The endpieces are 3 by 6 inches and the hole in each piece, which is $\frac{5}{8}$ inch in diameter, accommodates a drinking fountain tube or the tail of the rat. Fourteen steel rods, $6\frac{1}{2}$ inches long and $\frac{1}{8}$ inch in diameter, set approximately $\frac{1}{2}$ inch apart, constitute the floor and sides of the cage. These fit into slots drilled in one endpiece. Holes in corresponding positions in the other endpiece are drilled through and threaded to accommodate small brass screws. The enclosed space, $1\frac{3}{4}$ inches wide and $1\frac{3}{4}$ inches high, accommodates a rat which weighs 200 grams. Additional holes and slots may be made in the endpieces so that the rods may be placed to fit larger or smaller rats. The food cup, which is placed under an opening in the floor, has been satisfactory. To move a rat in or out of the cage it is necessary only to remove the screws at the ends of two or three rods and slide the rods through the openings.

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TECHNIQUES FOR THE COLLECTION OF LYMPH FROM THE LIVER, SMALL INTESTINE, OR THORACIC DUCT OF THE RAT

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THE following techniques for collection of lymph in the rat have been very satisfactory for periods of two or three days for lymph from the liver and for periods up to ten days for lymph from the intestine or from the thoracic duct. These methods have been useful in the study of lymph from the standpoint of the substances exchanged from the plasma to the lymph and of the materials contributed to the lymph from the intestine in the course of digestion and absorption of various foods.

Cannulation of the lymphatics of the liver is accomplished in rats weighing 150 to 300 grams. The rats are anesthetized with ether. Through a midline incision the liver is reflected upward to the right and the stomach and duodenum are reflected to the left; this exposes the hepatogastric and hepatoduodenal ligaments. Evans blue dye, 0.1 ml. of a 0.5 per cent solution, is injected through a fine hypodermic needle into the liver. Within a minute blue lymph may be seen in the lymphatics passing through the hepatogastric ligament. After the worker has acquired a little experience the clear lymphatics of the liver are easily recognized and the injection of dye is no longer necessary.

With the use of dissecting glasses, a blunt dissection with curved mosquito forceps proved the most practical method for isolation of the lymphatics. The inferior vena cava is dissected free above the right renal vein and a sharp 13 gauge needle is passed under the vena cava in line with the lymphatic vessel of the liver and through the abdominal wall. A plastic tube, Transflex, 1 or 1.5 mm. in diameter, with beveled ends, is filled with a dilute solution of heparin and passed through the needle to the outside. The needle is then removed. As much of the lymphatic as is exposed, usually 3 to 5 mm., is dissected free and ligated as far distally as possible. A small longitudinal opening is made in the lymphatic with the cutting edge of a 27 gauge hypodermic needle. One of the beveled tips of the plastic tubing is then passed into the lymphatic as far as possible and tied firmly in place (Fig. 1). A second ligature is placed near the vena cava so that the tubing is held in line with the direction of the lymphatic. Any accessory lymphatics in this region may be included in the ligature and, since they anastomose freely, their flow is diverted to the cannulated lymphatic. Occasionally a small lymphatic from the duodenum may deliver cloudy lymph to the lymphatic vessel in the liver; this can be prevented by ligation of the small

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lymphatic connection. In the rat which has been fed a diet containing fat, the lymph from the liver remains clear and contamination with intestinal lymph is easily recognized.

The lymph begins to flow immediately through the tubes, and after the incision is repaired the animal is placed in the cage described in another paper.[1] The lymph is collected in a graduated centrifuge tube from the plastic tube, the free end of which is passed through a small opening in a rubber cap on the centrifuge tube. The plastic tube acts as a siphon and delivers the lymph best at 5 to 10 cm. below the level of the rat.

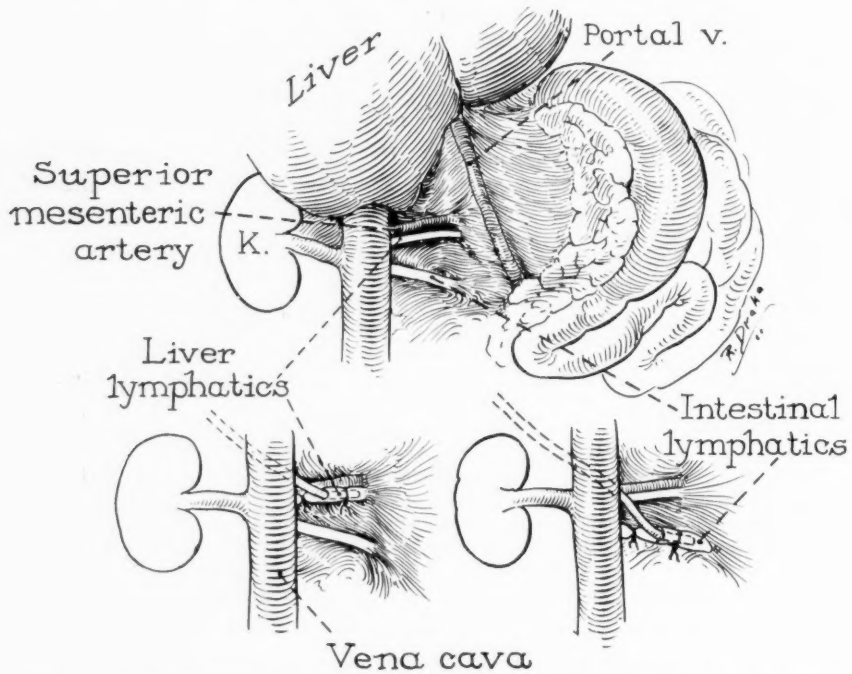

Fig. 1.—Structures involved in cannulation of a lymphatic of the liver and of an intestinal lymphatic.

Cannulation of the intestinal lymphatic which drains most of the small intestine is accomplished in a manner similar to that just described. In the rat which has been fed a meal that contains fat the intestinal lymphatics are easily recognized because they appear in the lower portion of the hepatoduodenal ligament. Evans blue dye injected into the substance of the intestine also stains the intestinal lymph blue. There usually are accessory intestinal lymphatics which need to be ligated so that all the intestinal lymph may be collected through the main channel.

Cannulation of the thoracic duct is accomplished in the etherized rat through an incision just distal to the last rib, extending from the midline anteriorly to the medial border of the left quadratus lumborum muscle posteriorly (Fig. 2). A small gauze pack, placed in such a way that it pushes the stomach, liver, and intestines back and to the right, exposes the left portion of the dia-

phragm, the aorta, and the left adrenal gland and kidney. A self-containing mastoid retractor is used to retract the kidney distally and to hold the incision open. With the use of dissecting glasses a small opening is made in the peritoneum over the quadratus lumborum, approximately 0.5 cm. cephalad to the superior suprarenal artery. The veins and peritoneum are dissected super-

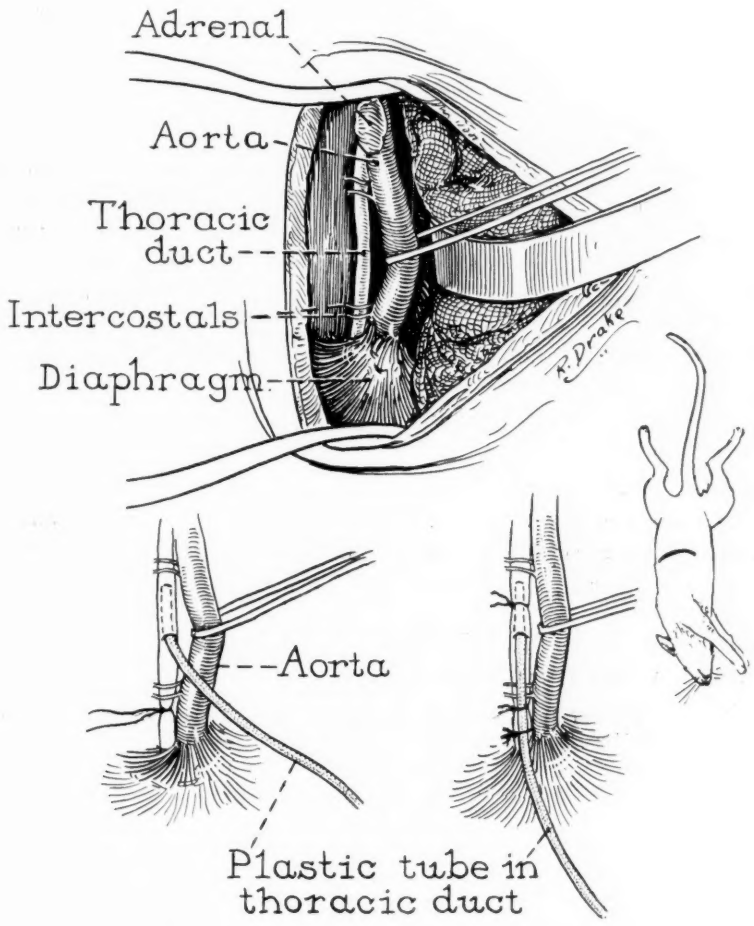

Fig. 2.—Method of cannulation of the thoracic duct.

ficially and retracted to the right until the aorta is exposed. The aorta in this region is freed of attached tissue until the left subcostal artery is exposed. The thoracic duct is visible just posterior to the aorta. It is 1 to 2 mm. in diameter and is embedded in loose connective tissue and fat. The thoracic duct is exposed for a length of 5 to 8 mm. by gentle blunt dissection, and a ligature is passed around it at the upper end of the exposed portion just caudad to the subcostal artery. A second ligature is placed 3 to 5 mm. caudad to the first. A sharp 13 gauge needle is passed through the abdomen at approximately the level of the xiphoid process and the beveled plastic tubes, 1.5 mm. in diameter, which contain

a dilute solution of heparin, are threaded through to the site of cannulation. A small longitudinal opening is made in the left anterior surface of the thoracic duct with the cutting edge of a 27 gauge hypodermic needle. This opening may be enlarged with a probe. A beveled end of the plastic tube is then slipped gently into the duct for 5 to 10 mm. and tied firmly in place with the second ligature. The first ligature is tightened about the cannula and the ends of both ligatures are tied together for further security. The tube is arranged so that it will lie relatively straight in the duct and yet will curve along the diaphragm before it passes through the abdominal wall. The gauze pack is removed, the viscera are replaced in proper position, and the incision is closed.

After operation the rats are placed in cages constructed according to the description previously mentioned. These cages are small in order to prevent the rat from turning around, but they do permit some forward and backward movement. The volume of lymph obtained varies somewhat with the dietary and fluid balance of the animal. For normal rats which weigh 200 grams and receive a mixed diet and water as desired, approximately 5 c.c. of clear hepatic lymph are collected each twenty-four hours for two or three days, when dislodgment or clotting within the cannula terminates the flow of lymph. In similar rats under similar conditions approximately 20 c.c. of intestinal lymph are collected every twenty-four hours and the flow of lymph is usually continuous up to about ten days. From the thoracic duct approximately 25 c.c. of lymph are collected each twenty-four hours for periods up to ten or more days. This figure is slightly greater than that reported by Reinhardt[2] for nonfasting adult rats whose thoracic ducts were cannulated by a different approach while they were under the effects of sodium pentobarbital anesthesia.

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2. Reinhardt, W. O.: Rate of Flow and Cell Count of Rat Thoracic Duct Lymph, Proc. Soc. Exper. Biol. & Med. **58**: 123, 1945.